University of Alberta

Characterization of Ovarian Dysfunction and Cardiovascular Disease Risk in the Female JCR:LA-*cp/cp* Rat : a Putative Model of Polycystic Ovary Syndrome

by

©

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Abstract

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, oligo-/anovulation and polycystic ovaries, and is highly associated with cardiometabolic disorders. Dyslipidemia is the most common metabolic disorder in PCOS, however the etiological mechanisms involved are remain unclear, partly due to a lack of suitable animal models of PCOS. The aim of this thesis was to establish the JCR:LA-*cp/cp* rat as a model of spontaneous PCOS, and to evaluate cardiovascular disease (CVD) risk factors in this model. The *cp/cp* genotype demonstrated increased testosterone concentrations, oligo-ovulation and cystic ovaries. The PCOS *cp/cp* rats developed obesity, insulin resistance and dyslipidemia, including increased triglyceride (TG), total cholesterol and apolipoprotein B48 (apoB48) concentrations. PCOS *cp/cp* rats had a greater postprandial plasma TG and apoB48 response following an 'oral fat challenge'. Consistently, intestinal lymph analysis showed that PCOS *cp/cp* animals secreted double the chylomicron particles in both the fasted and postprandial state. Collectively the JCR:LA-*cp/cp* rat is a useful model to study PCOS associated with CVD risk.

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List of Abbreviations

AMH	antiMüllerian hormone
ApoB48	apolipoprotein B48
AUC	area under the curve
BMI	body mass index
CIMT	carotid intima-medial thickness
CM	chylomicron
CMR	chylomicron remnant
CRP	C-reactive protein
CVD	cardiovascular disease
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
E_2	estradiol
ERK1/2 MAPK	extracellular signal-regulated kinase 1/2 mitogen activated protein kinase
ET-1	endothelin-1
EV	estradiol valerate
FFA	free fatty acid
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
HDL	high density lipoprotein
iAUC	incremental area under the curve
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IR	insulin resistance
IRS-1	insulin receptor substrate-1
IVRT	isovolumetric relaxation time
LDL	low density lipoprotein
LH	luteinizing hormone
LPL	lipoprotein lipase
LV	left ventricular
mTOR	mammalian target of rapamycin
MTP	microsomal triglyceride protein
MTT	meal tolerance test
NCAH	nonclassic adrenal hyperplasia
NO	nitric oxide
OFC	oral fat challenge

OFTT	oral fat tolerance test
PAI-1	plasminogen activator inhibitor type 1
PCOS	polycystic ovary syndrome
PKA-HSL	protein kinase a-hormone sensitive lipase complex
PKCξ	protein kinase Cξ
S6K	ribosomal S6-kinase
SHBG	sex hormone binding globulin
SR-B1	scavenger receptor-b1
T	testosterone
TC	total cholesterol
TG	triglyceride
TP	testosterone propionate
VLDL	very low density lipoprotein

1. Literature Review

1.1 Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women, affecting 5-10% of women in their reproductive years (Norman *et al* 2007, Homburg 2008). PCOS is characterized by hyperandrogenism, oligo-/anovulation and polycystic ovaries. In recent years, PCOS has become highly associated with the metabolic syndrome, cardiovascular and inflammatory risk markers (Ehrmann *et al* 2005, Franks 1995, Dunaif 1997, Fogel *et al* 2001, Escobar-Morreale *et al* 2004, Norman *et al* 2007). There is now an emerging public health concern regarding the long-term health complications of PCOS, such as type 2 diabetes and cardiovascular disease (CVD) (Franks 1995, Norman *et al* 2007). Since Stein and Leventhal first described the syndrome in 1935 (Stein *et al* 1935), the quest of many researchers has been to identify the etiological mechanisms associated with the onset of PCOS. Despite the prevalence of PCOS, the early etiology and long term health risks of this syndrome remain unclear. Emerging evidence is showing that PCOS is a complex syndrome, involving multiple etiological factors; which leads to a vast heterogeneity in clinical and biochemical symptoms (Franks 1995, Norman *et al* 2007). The heterogeneity of clinical symptoms has further complicated the definition of the clinical features of PCOS. Investigations into the metabolic and CVD risk factors associated with PCOS have been extensive (Cussons *et al* 2006, 2008), however it is still unclear how these risk factors relate to the early development and long term health outcomes in women with the syndrome. Therefore the development of suitable animal models of PCOS has become a key area of research in order to further understand the PCOS condition in relation to metabolic risks associated with type 2 diabetes and CVD. The focus of this literature review is to provide a background into the metabolic risk factors associated with CVD in PCOS and the characteristics of current animal models available to study these factors in PCOS.

1.2 Regulation of Endocrine Profile in the Normal Menstrual Cycle

The human menstrual cycle, including the follicular phase and luteal phase, is mainly regulated by the hypothalamic-pituitary-ovarian axis (Fig 1-1). Gonadotropin-releasing hormone (GnRH) secreted by the hyperthalamus stimulates the release of gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which then act on the ovaries (Yen, 1999). The ovarian follicle is composed of two distinct cellular compartments, the theca and granulosa cells (Fig. 1-2). Theca cells respond to LH and synthesize androstenedione and testosterine (T). Androstenedione is then converted to estrone in the granulosa cells by the action of aromatase, which is under the influence of FSH (Balen 2004) (Fig. 1-3). The hormone profile during a normal menstrual cycle is shown in Fig. 1-4. The first half the cycle is referred to as the follicular phase and is characterized by a progressive increase in circulating levels of estradiol secreted by the developing dominant follicle. The increased estradiol level has a positive feedback on both the hypothalamic GnRH secretion and the pituitary LH and FSH secretion. At late follicular phase, the LH and FSH surges are associated with the peak estradiol levels, which cause ovulation and the onset of the luteal phase. The hall mark of the luteal phase is the shift from the estrogen-dominated follicular phase to corpora-progesterone dominance. The luteinized theca-granulosa cells after ovulation form corpora lutea and begin to synthesize large amounts of progesterone and a small amount of estradiol. Unless implantation occurs, the corpora lutea degenerate and become corpora albicans, with a prompt linear decline in circulating progesterone and estradiol, which causes menstruation and the start of a new menstrual cycle (Yen, 1999).

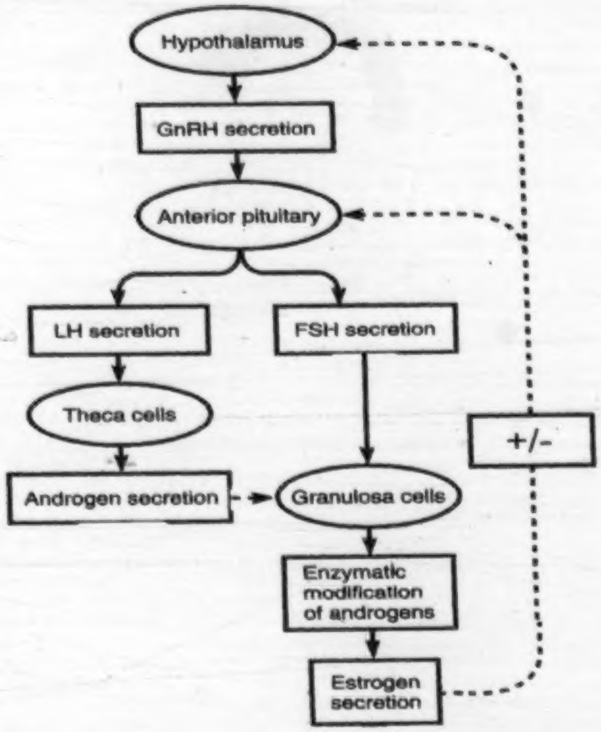

Figure 1-1. Regulation of hormone secretion by the hypothalamic-pituitary-ovarian axis. During the early to mid-follicular phase, the relatively low plasma estradiol levels provide negative feedback for the release of GnRH, FSH, and LH. During the late follicular phase, the relatively high plasma estradiol levels provide positive feedback for the release of GnRH, FSH, and LH.

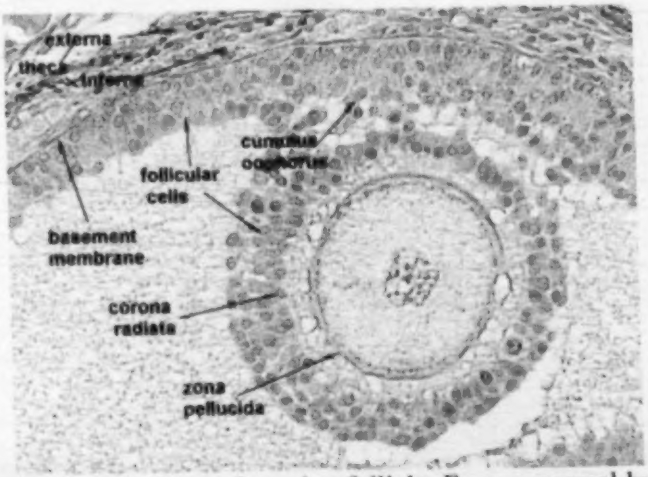

Figure 1-2. Histology of ovarian follicle. From a normal human ovary. From http://www.octc.kctcs.edu/gcaplan/anat2/notes/Notes2%20fema le%20reproductive%20anatomy.htm.

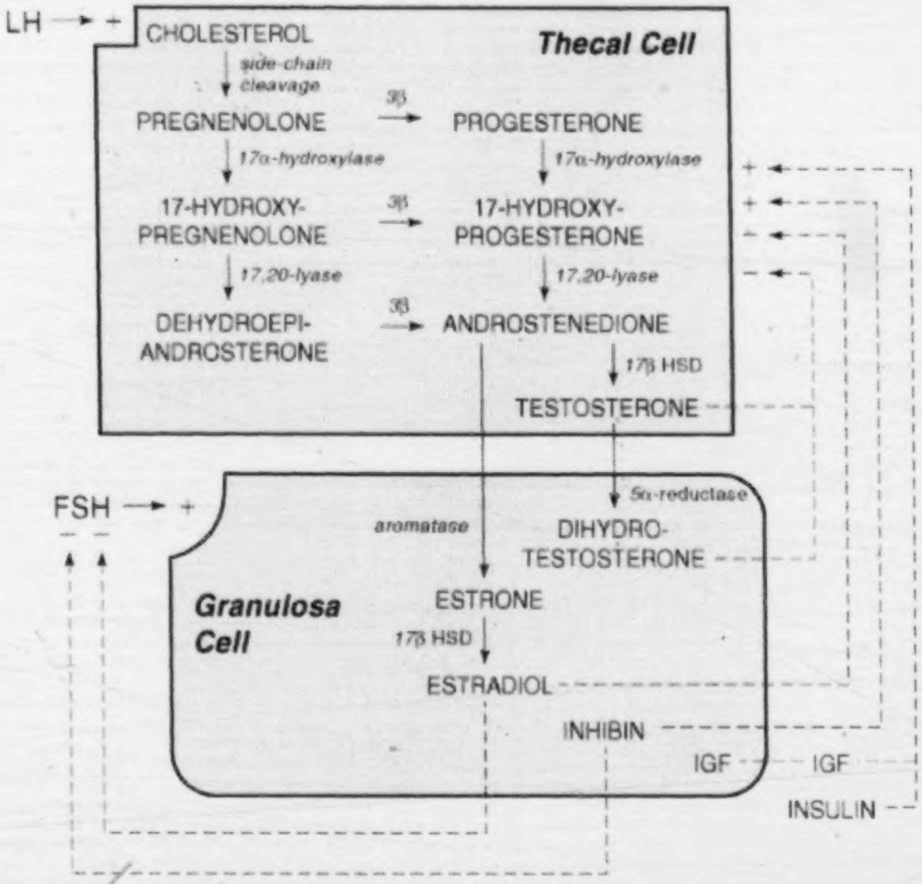

Figure 1-3. The two-cell, two-gonadotropin concept of follicle estrogen biosynthesis. Luteinizing hormone (LH) stimulates theca cells to express cholesterol side-chain cleavage cytochrome P450, 3-hydroxysteroid dehydrogenase (3β), and 17-hydroxylase/C17–20 lyase cytochrome P450. The theca cells can then synthesize androstenedione from cholesterol. The androstenedione diffuses across the basal lamina into the granulosa cells. Follicle-stimulating hormone (FSH) stimulates the expression of aromatase cytochrome P450 and 17-hydroxysteroid dehydrogenase (17β-HSD) in the granulose cell. The granulosa cells can then metabolize the androstenedione to estradiol. Androgens and estrogens are negative modulators of LH effects, while IGFs play a positive modulator role. Insulin also augments LH-stimulated androgen production, either via its own receptors or via IGF-1 receptors. Inhibin promotes androgen synthesis. Both estradiol (E_2) and inhibin inhibit FSH bioactivity (From Gardner *et al* 2007).

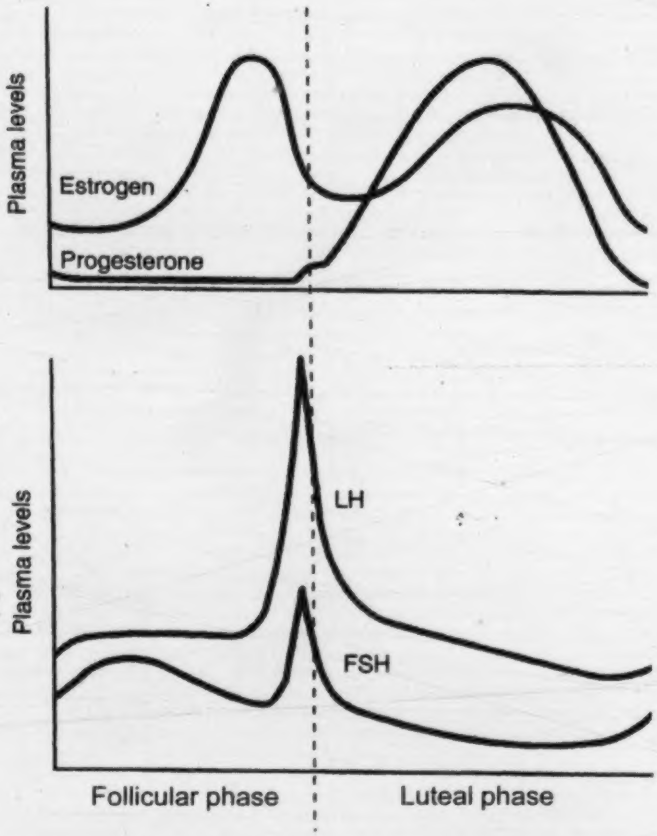

Figure 1-4. Hormone profile during a normal menstrual cycle.
The verticle dashed line indicates the occurrence of ovulation.

1.3 Characteristic Features of PCOS

The primary characteristics of PCOS are associated with changes in ovarian morphology, endocrine hormone profile and metabolic abnormalities.

Ovarian Morphology

Current data suggests that polycystic ovaries, detected by transvaginal ultrasonography, are found in approximately 75% of women with a clinical diagnosis of PCOS (Azziz *et al* 2006). In those women with PCOS, the primary ovarian morphological characteristics include bilateral enlargement of the ovaries with a smooth and thickened avascular capsule. The stromal area of the ovaries is increased and contains multiple medium-sized follicles ($\geq$12 follicles of 2-9 mm in diameter) (Fig. 1-5). Histologically, these follicles are atretic and/or cystic and lined with degenerating granulosa cells. Follicular development appears to be arrested at the small antral stage with hyperplasia of the androgen-producing thecal layers. The frequency of corpora lutea is significantly reduced indicating an anovulatory state (Franks 1995, Jonard *et al* 2003, Balen *et al* 2003, Rotterdam Consensus 2004, Fulghesu *et al* 2007).

Endocrine Hormone Profile

Ovarian derived hyperandrogenemia is considered a key etiological trait of PCOS (Azziz *et al* 2006a). Indeed, approximately 60-80% of women with PCOS have high concentrations of androgens, predominantly attributable to testosterone (T), with mean concentrations ranging from 50% to 150% higher compared to non-PCOS subjects (Azziz *et al* 2006a, Franks 1989). Serum estradiol (17β-estradiol, E_2) concentrations in PCOS women are normal during early and mid-follicular phases of the ovarian cycle (Polson *et al* 1987). However, E_2 secretion at pre-ovulation and at the mid-luteal phase can be diminished leading to chronic anovulation. Luteinizing hormone (LH) pulse amplitude and frequency are increased, leading to a two to three-fold increase in 24-hour mean LH concentrations (Morales *et al* 1996). Follicle stimulating hormone (FSH) concentrations are decreased or unvarying, which is related to the dysfunction in folliculogenesis and follicular arrest (Brown 1978) (Fig. 1-6). The ratios of LH/FSH and T/E_2 are often increased. Hyperprolactinemia is also common in women with PCOS (Franks 1995). Accordingly, PCOS women clinically present with infertility, oligomenorrhea and symptoms of hyperandrogenism, including hirsutism, acne and male-pattern alopecia.

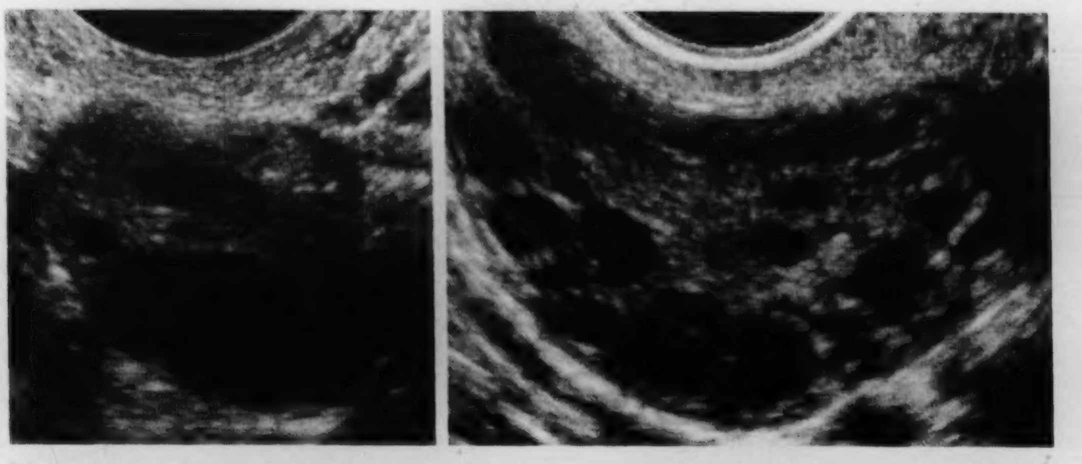

Figure 1-5. Normal (left) and polycystic ovary (right) shown by transvaginal ultrasonography during the follicular phase of a menstrual cycle. The fluid-filled antrum of a developing follicle appears as a dark circle. Compared with a normal ovary, the polycystic ovary is typically enlarged and contains an abnormally increased number of medium-sized follicles (Norman *et al* 2007).

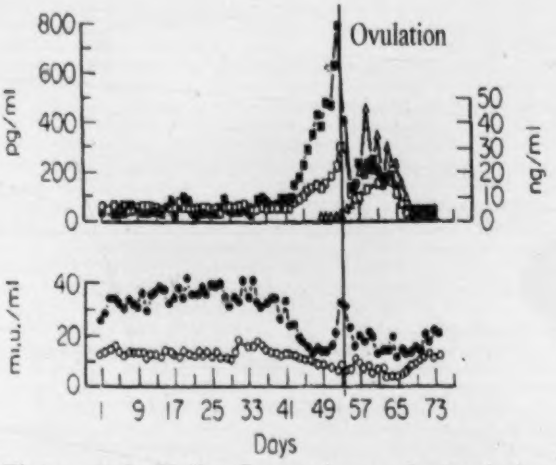

Figure 1-6. Daily fluctuations of circulating estrogens and progesterone (top: ■,estradiol; □,estrone; Δ,progesterone) and gonadotropin (bottom: ●,LH; ○,FSH) monitored during a period of 74 days in a patient with PCOS. Within the period of study, spontaneous ovulation occurred with concomitant reduction of LH and androgen levels during the luteal phase (Yen, 1999).

Metabolic Abnormalities

In recent years PCOS has become associated with the metabolic syndrome. It is estimated that approximately 45% of women with PCOS have the metabolic syndrome, and more than 90% of PCOS women have at least one metabolic abnormality, including insulin resistance (IR) and compensative hyperinsulinemia, visceral obesity and dyslipidemia (Essah *et al* 2006). Collectively, these risk factors appear to pre-dispose individuals to type 2 diabetes and CVD, and will be discussed below in further detail (Franks 1995, Rotterdam Consensus 2004, Orio *et al* 2006, Norman *et al* 2007).

1.4 Diagnostic Criteria of PCOS

Since the phenotype and severity of symptoms vary amongst individuals with PCOS, (Balen *et al* 1995, Elting *et al* 2000), the diagnostic criteria is a controversial issue (Balen *et al* 2002, Azziz 2006b, Franks 2006a, Barth *et al* 2007). There have been three diagnostic criteria for PCOS since 1990 (Zawadski *et al* 1992, Rotterdam Consensus 2004, Azziz *et al* 2006a), (Table 1-1). The most recent criteria for diagnosing PCOS were proposed by the Androgen Excess Society (AES) task force in 2006 (Azziz *et al* 2006a). These criteria addressed PCOS as a disorder of androgen excess or hyperandrogenism with exclusion of other androgen excess or related disorders, such as 21-hydroxylase deficient nonclassic adrenal hyperplasia (NCAH), hyperprolactinemia and thyroid abnormalities. The task force recognized nine different phenotypes of PCOS based on the presentation of four key features (Table 1-2, Azziz *et al* 2006a):

1. hyperandrogenemia (predominantly attributable to T)
2. hirsutism
3. ovulatory dysfunction (oligo-/anovulation)
4. polycystic ovaries (at least one ovary demonstrates an ovarian volume of greater than 10 cm^3 and/or 12 or more follicles measuring 2–9 mm in diameter).

In addition to these criteria, the task force also emphasized the associated metabolic disorders, including obesity, IR and dyslipidemia, which are highly prevalent in women with PCOS (Azziz *et al* 2006a). Therefore, it is now considered mandatory to evaluate metabolic dysfunction in all PCOS patients, regardless of body weight. This mandate includes fasting plasma glucose and insulin concentrations, and postprandial response to a glucose tolerance test (2hr). The assessment of lipid profile assessment has also been recommended (Trivax *et al* 2007, Salley *et al* 2007).

Table 1-1. Diagnostic criteria of polycystic ovary syndrome.

NIH (1990)	To include all of the following: 1. Hyperandrogenism and/or hyperandrogenemia 2. Oligo-ovulation 3. Exclusion of related disorders
ESHRE/ARMS (Rotterdam 2003)	To include two of the following and exclude related disorders 1. Oligo- or anovulation 2. Clinical and/or biochemical signs of hyperandrogenism 3. Polycystic ovaries
AES (2006)	To include all of the following 1. Hyperandrogenism (hirsutism and/or hyperandrogenemia) 2. Ovarian dysfunction (oligo-anovulation and/or polycystic ovaries) 3. Exclusion of androgen excess or related disorders

NIH, National Institutes of Health; ESHRE/ARMS, European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine; AES, Androgen Excess Society.

Table 1-2. All possible phenotypes based on the presence or absence of oligoanovulation, hyperandrogenemia, hirsutism, and polycystic ovaries.

Feature	Potential phenogypes															
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Hyperandrogenemia	+	+	+	+	-	-	+	-	+	-	+	-	-	-	+	-
Hirsutism	+	+	-	-	+	+	+	+	-	-	+	-	-	+	-	-
Oligoanovulation	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-
Polycystic ovaries	+	-	+	-	+	-	+	+	+	+	-	+	-	-	-	-
NIH 1990 Criteria	√	√	√	√	√	√										
Rotterdam 2003	√	√	√	√	√	√	√	√	√	√						
AES 2006 Criteria	√	√	√	√	√	√	√	√	√							

+, Presence, -, absence. From Azziz *et al* 2006.

1.5 Etiology and Pathophysiology of PCOS

The precise etiology of PCOS remains unclear, yet the heterogeneity of the clinical and biochemical features of the syndrome suggests that several factors may contribute to the pathophysiology of this syndrome. Indeed there is increasing evidence for a genetic component in the development of PCOS based on the familial clustering of cases. For example, the prevalence of PCOS in the first-degree relatives of those with PCOS is five to six times higher than in the general population (Kahsar-Miller *et al* 2001, Amato *et al* 2004). However, the mode of inheritance of PCOS is not clear, which may be autosomal dominant, but is much more likely to be oligogenic or polygenic (Franks *et al* 1997, 2006b). Candidate genes include those that are involved in the regulation of the hypothalamic–pituitary–ovarian axis, androgen synthesis pathways, insulin secretion or action, or folliculogenesis (Ehrmann 2005). In addition to the genetic components, environmental factors are also implicated in the development of PCOS, such as prenatal exposure to excess androgens, which is one of the etiological hypotheses of PCOS induction (Abbott *et al* 2005). Therefore, the etiology of PCOS appears to be polygenic and multifactorial (Amato *et al* 2004).

There are four main interrelated perturbances that have been identified to be involved in the pathophysiology of PCOS: ovarian androgen excess; abnormal ovarian morphology; hyperstimulation of LH; IR and hyperinsulinemia (Norman *et al* 2007).

1.5.1 Ovarian Androgen Excess

PCOS has been recognized as a disorder of ovarian androgen excess or hyperandrogenism. In 60-80% of women with PCOS, increased circulating T concentration is a principle biochemical feature (Azziz *et al* 2006a). Follicles in PCOS have a thickened thecal layer, and both in vivo and in vitro (in cultured theca cells) studies have confirmed that the theca cells from affected women produce more T either under basal conditions or in response to LH stimulation, compared to those from normal women (Nelson *et al* 1999, 2001, Wickenheisser *et al* 2006). Studies have also demonstrated that various enzymatic actions involved in androgen production are up-regulated in polycystic ovaries, including P450c17 (17-hydroxylase and 17,20-lyase), 3-beta-hydroxysteroid dehydrogenase type II (3β) and side-chain cleavage enzyme, while the activity of aromatase, which converts androgens to estrogens, is decreased. (Ehrmann *et al* 1995, Norman *et al* 2007, Diamanti-Kandarakis 2008) (Fig. 1-5). Therefore, primary defects of steroidogenesis were proposed as one of the etiological hypotheses of PCOS (Zhang *et al* 1995). However, it is still uncertain whether the dysregulation of these enzymes is of genetic origin or due to the improper regulation of gonaditropins and/or insulin effects on these pathways (Escobar-Morreale *et al* 2005).

1.5.2 Abnormal Ovarian Morphology

In women with PCOS, approximately two to six times more primary, secondary and early antral follicles are present in the ovaries compared to those from control women (Webber *et al* 2003, Maciel *et al* 2004). Follicular development appears to be arrested and antral follicles only reach a diameter of 2-9mm (Webber *et al* 2003, Maciel *et al* 2004). These features suggest that there are underlying disorders of folliculogenesis, which may include increased follicular recruitment and an arrest of follicular development at this small antral stage. It has been suggested that the disturbed follicular maturation may be due to the abnormal endocrine environment (Norman *et al* 2007). A certain amount of intra-ovarian androgens are necessary for early follicular development. However, excess androgens can lead to poor follicle maturation and follicular atresia (Jonard *et al* 2004). Insulin and LH appear to be involved in the development of polycystic ovaries or follicular mal-development (Franks *et al* 1998). Some researchers have proposed that there may be an intrinsic abnormality within the follicle of the affected ovaries, such as a genetic defect that facilitates follicular growth and/or androgen secretion (Webber *et al* 2003).

1.5.3 Hypersecretion of LH

Approximately 40-60% of women with PCOS manifest LH hypersecretion (Taylor *et al* 1997), as demonstrated by increased amplitude and frequency of LH pulses (Waldstreicher *et al* 1988). Increased LH concentrations and elevated ratio of LH/FSH favor the overproduction of androgens in the ovarian theca cells (Fig. 1-5). It is well known that the production and secretion of pituitary LH and FSH are partly stimulated by the hypothalamic gonadotropin-releasing hormone (GnRH). Moreover, changes in the GnRH pulsatility (frequency of pulses) may further alter the ratio of LH/FSH. When the GnRH pulsatility is rapid, LH secretion predominates and the ratio of LH/FSH is increased (Haisenleder *et al* 1991). However, abnormal GnRH secretion may also be attributed to intrinsic neuroendocrine abnormalities or secondary to hyperandrogenism

(Barnes 1985). Other evidence suggests that the hypersecretion of LH may also be due to impaired sensitivity of the hypothalamus to progesterone, a hormone that has been thought to suppress the GnRH pulsatility (Pastor *et al* 1998, Eagleson *et al* 2000). In addition to the factors mentioned above, inhibin B is also involved in regulating FSH production and activity (Fig. 1-5). Inhibin B is synthesized by the granulosa cells in women. Studies have shown that circulating inhibin B levels are increased in PCOS women (Anderson *et al* 1998), and that inhibin B is capable of negatively regulating FSH secretion and bioactivity, which may indirectly contribute to LH predomination (Tsilchorozidou *et al* 2004).

1.5.4 Insulin Resistance and Hyperinsulinemia

Insulin resistance and compensatory hyperinsulinemia has been recognized as an important factor involved in the pathogenesis of PCOS. Indeed approximately 80% of obese women and 30–40% of lean women with PCOS have IR (Dunaif 1997). Insulin acts directly and indirectly to increase endogenous androgen concentrations through multiple sites (Table 3), which include the following:

a. Insulin increases T production as a result of enhanced amplitude of serum LH pulses (Nestler *et al* 1996, 1997) and the synergized effect of LH on the ovarian theca cells (Bergh *et al* 1993).
b. Insulin inhibits the hepatic synthesis of sex hormone binding globulin (SHBG) to increase serum free T concentrations (Nestler *et al* 1996).
c. Insulin decreases the synthesis of insulin-like growth factor binding protein-1 (IGFBP-1), resulting in increased bioavailability of IGF-1 and 2, which are important regulators of ovarian follicular maturation and steroidogenesis (De Leo *et al* 2000, Adashi 1993).
d. Insulin increases endogenous androgen concentrations by upregulating the

activity of cytochrome P450c17α enzyme, a key enzyme of ovarian and adrenal steroid hormone biosynthesis (Nestler *et al* 1996).

Table 1-3. Insulin effects related to ovarian function.

Insulin Effect	Organ
Directly stimulates steroidogenesis	Ovary
Acts synergistically with LH and FSH to stimulate steroidogenesis	Ovary
Stimulates 17-α-hydroxylase	Ovary
Stimulates or inhibits aromatase	Ovary, adipose tissue
Upregulates LH receptors	Ovary
Promotes ovarian growth and cyst formation (synergistically with LH and hCG)	Ovary
Downregulates insulin receptor	Ovary
Upregulates type 1 IGF receptors or hybrid insulin/type 1 IGF receptors	Ovary
Inhibits IGFBP-1 production	Ovary, liver
Inhibits SHBG production	Liver
Potentiates the effect of GnRH on LH and FSH	Hypothalanmus, pituitary

Adapted from Salehi *et al* 2004.

Although the cellular and molecular mechanisms of IR in PCOS remain uncertain, current evidence suggests that the major mechanism is a decrease in insulin sensitivity secondary to a post-binding abnormality in insulin receptor-mediated signal transduction (Dunaif *et al* 1995, Corbould *et al* 2005). An increase in serine phosphorylation of the insulin receptor or insulin receptor substrate (IRS)-1 inhibits the metabolic signaling (Dunaif *et al* 1995, Corbould *et al* 2005). This defect may impair glucose transport and affects 50% of women with PCOS (Dunaif 1997). It is recognized that insulin has both metabolic (carbohydrate metabolism) and mitogenic (growth) effects, and increased serine phosphorylation affects only metabolic pathways. Indeed, selective defects of insulin sensitivity in PCOS have been observed in several studies (Book *et al* 1999, Wu *et al* 2003). In different tissues, some are highly insulin resistant (i.e. skeletal muscle), while others are insulin sensitive (i.e. adrenal and ovary). In affected tissues, metabolic pathways are generally resistant to stimulation by insulin, but mitogenic or steroidogenic pathways are not (Diamanti-Kandarakis *et al* 2006). Furthermore, Baillargeon and coworkers (2004, 2006) have observed that insulin-sensitizing compounds/intervention improve the symptoms of PCOS (increasing ovulatory frequency and amelioration of hyperandrogenemia). This is also apparent even in non-obese PCOS women with clinically normal indices of insulin sensitivity. Consequently, it has been proposed that intrinsic defects, leading to selective and tissue-specific increases in insulin sensitivity in the ovarian androgenic pathway (the mitogenic pathway), may be one of the etiologies of PCOS. One study by Zhang *et al* (1995) has shown that serine phosphorylation increases activity of P450c17 in both the ovary and adrenal gland, thus promoting androgen synthesis. It is possible that a single defect (serine phosphorylation) can produce both IR and hyperandrogenism in a subgroup of PCOS patients (Tsilchorozidou *et al* 2004). Although, hyperinsulinemia is believed to play a crucial role in PCOS, its relationship to hyperandrogenism remains unclear and requires further research (Tsilchorozidou *et al* 2004). Current evidence seems to support the concept that hyperinsulinemia is the primary defect of PCOS induction. Studies have shown that insulin sensitizing treatment in both lean and obese PCOS women can ameliorate hyperandrogenemia (Baillargeon *et al* 2006, Velazquez *et al* 1994), while anti-androgen treatment does not improve the hyperinsulinemic state or alter insulin sensitivity (Dunaif *et al* 1990, Nagamani *et al* 1986, Diamanti-Kandarakis *et al* 1995).

1.6 Cardiovascular Disease and PCOS

1.6.1 Cardiovascular Disease Risk Factors in PCOS

Women with PCOS often demonstrate features of the metabolic syndrome, such as IR, dyslipidemia and obesity (Norman *et al* 2007). The prevalence of the metabolic syndrome in PCOS women is approximately 45%, 2-fold higher than age-matched women in the general population (Apridonidze *et al* 2005, Ford *et al* 2002, Legro 2002a). Prospective studies have shown that the metabolic syndrome is associated with a 2-fold increased risk of atherosclerotic vascular disease compared to individuals without the metabolic syndrome (Grundy *et al* 2005). The National Cholesterol Education Projects Adult Treatment Panel III guidelines also recognize the metabolic syndrome as a major CVD risk factor (NCEP 2001). It has been hypothesized that the metabolic disturbances in PCOS interact and mediate inflammation, oxidative stress and endothelial dysfunction, leading to premature clinical cardiovascular disease (Cussons *et al* 2006) (Table 1-7).

1.6.1.1 Insulin Resistance in PCOS

Insulin resistance is believed to play a crucial role in the pathogenesis of PCOS and the metabolic syndrome (Dunaif *et al* 1989, Diamanti-Kandarakis 2008). Both obese and non-obese women with PCOS are more insulin-resistant and hyperinsulinemic than age- and weight-matched controls (Dunaif *et al* 1989, 1996). About 60–65% of U.S. women with PCOS have peripheral IR, affecting mainly muscle and adipose tissue, and a compensatory hyperinsulinemia independent of obesity (Dunaif *et al* 1997). It is estimated that 40% of women with PCOS have either impaired glucose tolerance (IGT) or type 2 diabetes (Legro *et al* 1999, Dunaif 1995). Sam and Dunaif (2003) have proposed that IR is the most significant determinant of cardiovascular disease in PCOS patients (Balen *et al* 2005, Table 1-4). IR and compensatory hyperinsulinemia is a significant risk factor for development of hypertension, endothelial dysfunction and CVD (Sowers *et al* 1994, Despres *et al* 1996, Lamounier-Zepter *et al* 2006).

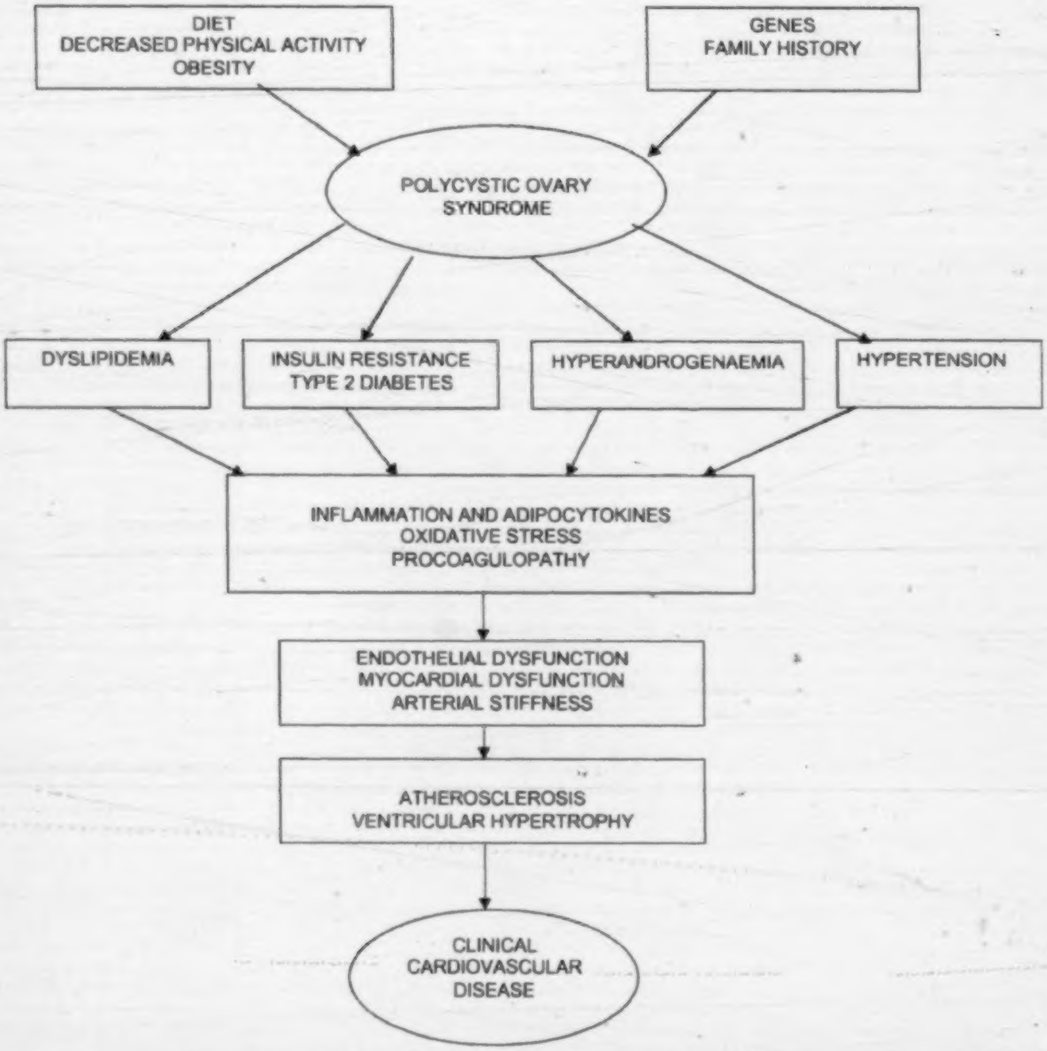

Figure 1-7. Hypothetical scheme for the pathogenesis of cardiovascular disease in PCOS. This figure summarizes potential pathways through which the cardiovascular risk factors associated with PCOS may translate into clinical cardiovascular disease. This scheme is hypothetical, however, and while supported by valid a priori studies requires examination and verification in prospective clinical outcome studies. From Cusson *et al* 2006.

PCOS has been shown to be associated with hypertension (Conway *et al* 1992, Talbott *et al* 1995, Elting *et al* 2001), and endothelial dysfunction (Paradisi *et al* 2001). Hyperinsulinemia may directly contribute to hypertension by increasing sympathetic nervous system activity and enhancing renal sodium retention (Sowers *et al* 1994, Reaven *et al* 1996). Hyperinsulinemia may also suppress the secretion of nitric oxide (NO) and other vasodilatory factors (Zeng *et al* 2000). In the insulin-resistant state, vascular endothelial dysfunction is the key factor leading to the development of atherogenesis and increased risk of cardiovascular events (Hsueh *et al* 2003). IR may decrease the production of endothelial NO, a vasodilator and increase vasoconstrictor endothelin-1 (ET-1) secretion, mediating endothelial dysfunction in PCOS (Diamanti-Kandarakis *et al* 2001).

Table 1-4. Cardiovascular disease risk factors related to insulin resistance in women with PCOS (based on varying diagnostic criteria).

Decreased cardiac systolic flow velocity (Dahlgren *et al* 1992)
Diastolic dysfunction (Yarali *et al* 2001)
Endothelial dysfunction (Paradisi *et al* 2001)
Increased vascular stiffness (Kelly *et al* 2002a)
Low-grade chronic inflammation (Kelly *et al* 2001)
Increased oxidative stress (Loverro *et al* 2002)
Altered circulating divalent cations (Muneyyirci-Delale *et al* 2001)
Altered hemostasis including impaired fibrinolysis (Polson *et al* 1988)
Increased tissue plasminogen activator antigen (Kelly *et al* 2002b)

From Balen *et al* 2005

1.6.1.2 Dyslipidemia in PCOS

Dyslipidemia is the most common metabolic disorder in PCOS, affecting up to 70% of women with the syndrome (Talbott *et al* 1998, Legro *et al* 2001). Although previous studies on dyslipidemia in PCOS report conflicting results, most have shown that PCOS is associated with an atherogenic lipid profile (Table 1-5). Dyslipidemia in PCOS is typically characterized by low high-density lipoprotein (HDL) cholesterol, elevated triglyceride (TG), total and low-density lipoprotein (LDL) cholesterol (Talbott *et al* 1995, Conway *et al* 1992), and increased total apolipoprotein B (apoB) concentrations (Macut *et al* 2001). Further to the dyslipidemia described in the fasted state, studies have reported that postprandial dyslipidemia is also prevalent in women with PCOS (Velazquez *et al* 2000, Bahceci *et al* 2007). Velazquez *et al* (2000) observed that women with PCOS had an elevated postprandial TG response following a high fat meal. Most recently, Bahceci and coworkers (2007) evaluated the postprandial lipid response to an oral fat tolerance test (OFTT) in patients with PCOS. They observed that the AUC_{TG} (AUC, area under the curve), AUC_{TC} (TC, total cholesterol), and AUC_{VLDL} (VLDL, very-low-density lipoprotein) were increased by 67%, 28% and 69%, respectively in PCOS patients compared to BMI- and age-matched controls. It is well established that postprandial lipemia, in particular the delayed clearance of chylomicrons (CMs), is a significant contributor to dyslipidemia and the development of atherosclerosis (Groot *et al* 1991, Zilversmit *et al* 1995, Twickler *et al* 2005). The pathogenesis of dyslipidemia in PCOS remains controversial due to a cluster of interrelated risk factors involved in PCOS. IR, obesity and hyperandrogenemia may all contribute to the development of dyslipidemia in PCOS (Diamanti-Kandarakis *et al* 2007). Figure 1-8 shows the possible mechanisms of dyslipidemia in PCOS.

Table 1-5. Summary of dyslipidemia in the fasted state in PCOS.

Author/Year	Number of PCOS	Lipid Pattern
Talbott/1995	206	↑TG, ↑TC, ↑LDL, ↓HDL
Robinson/1996	77	↓HDL
Meirow/1996	31	↑TG, ↑TC, ↑LDL
Diamanti-Kandarakis/1998	17	↑TG, ↑LDL, ↓HDL
Talbott/1998	244	↑TC, ↑LDL
Macut/2001	29	↑TG, ↑TC, ↑LDL, (-)HDL, ↑apoB,
Dejager/2001	31	(-)TG, ↓HDL
Legro/2001	195	↑TG, ↑TC, ↑LDL, ↑HDL
Orio/2004b	30	(-)TG, ↑TC, ↑LDL, ↓HDL
Yilmaz/2005	85	(-)TG, (-)TC, (-)LDL, ↓HDL
Bahceci/2007	20	↑TG, (-)TC, (-)LDL, (-)HDL, ↑apoB

Insulin resistance plays a significant role in the development of dyslipidemia in PCOS. It has been shown that PCOS women with IGT or type 2 diabetes have increased incidence of dyslipidemia (Legro *et al* 2001). In insulin-resistant states, VLDL is overproduced by the liver and the clearance of TG-rich particles is decreased, resulting in hypertriglyceridemia (Adeli *et al* 2001). Low HDL-cholesterol levels, a strong risk factor of coronary heart disease, have also been observed in women with PCOS, which are often related to hypertriglycridemia (Legro *et al* 1999b). Furthermore, lipoprotein lipase (LPL) activity is found to be decreased in the insulin-resistant state (Potts *et al* 1995), which may also be one mechanism involved in the development of dyslipidemia in PCOS women. Interestingly, evidence has demonstrated that increased intestinal lipoprotein secretion is associated with IR (Federico *et al* 2006, Haidari *et al* 2002), which has recently been confirmed in a human study. Duez *et al* (2006) showed that hyperinsulinemia was associated with increased production of intestinal apoB48-

containing lipoproteins in humans (using a primed constant (12 h) infusion of deuterium-labeled leucine). These results emphasize the positive role of the intestine in the regulation of lipid/lipoprotein metabolism. However, the regulation of intestinal lipid metabolism has not been investigated in PCOS, but may be a significant contributor to dyslipidemia based on the recent observation of postprandial dyslipidemia in PCOS.

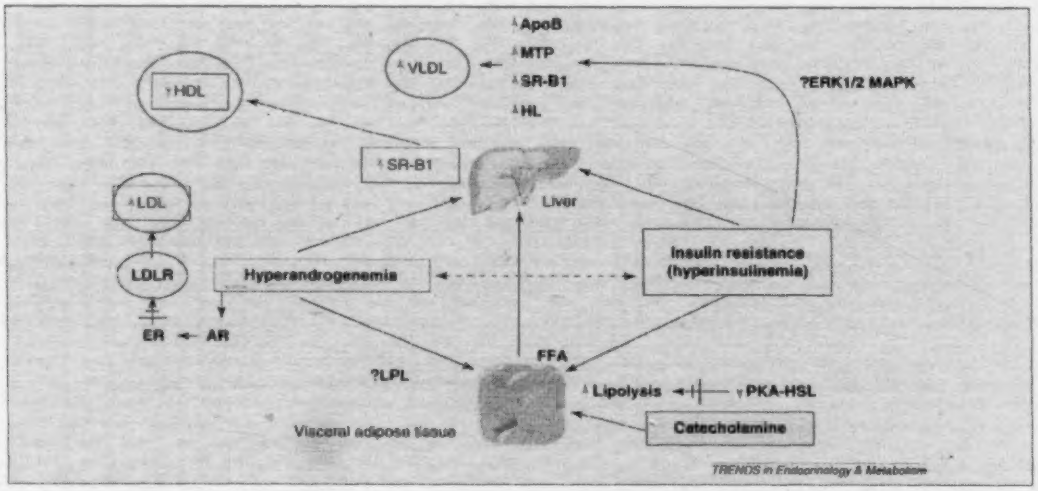

Figure 1-8. Possible mechanisms of dyslipidemia in PCOS. Within adipocytes, insulin resistance and hyperandrogenemia result in increased catecholamine-induced lipolysis and release of fatty acids into circulation. Increased free fatty acid flux to the liver stimulates the assembly and secretion of VLDL resulting in hypertriglyceridemia. The main serum lipid abnormalities in PCOS are indicated in red circles. Broken arrow represents potential interaction, ↑activation; ↓deactivation; ≠, inhibition. Abbreviations: ApoB, apolipoprotein B; AR, androgen receptor; ER, estrogen receptor; ERK1/2 MAPK, extracellular signal-regulated kinase 1/2 mitogenactivated protein kinase; FFA, free fatty acids; HL, hepatic lipase; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; MTP, microsomal triglyceride protein; PKA-HSL, protein kinase a-hormone sensitive lipase complex; SR-B1, scavenger receptor-b1; VLDL, very low density lipoprotein. From Diamanti-Kandarakis *et al* 2007.

In terms of obesity, overproduction of VLDL particles and defective lipoprotein lipase (LPL)-mediated lipolysis has been shown to contribute to dyslipidemia (Diamanti-Kandarakis *et al* 2007). Both obese and non-obese women with PCOS tend to have a centripetal or visceral pattern of body fat distribution. Yildirim *et al* (2003) found that visceral fat volume was highly associated with dyslipidemia, including hypertriglyceridemia and hypercholesterolemia. Visceral fat tissue can recycle fatty acids more rapidly through lipolysis, and release higher concentrations of free fatty acids (FFA) into the circulation compared to peripheral fat. Therefore, it has been hypothesized that visceral fat may directly mediate hepatic dyslipidemia during obesity (Matsuzawa *et al* 1995). Moreover, obesity is positively associated with IR, the latter being regarded as the main cause of the lipidemic aberrations in the obese PCOS phenotype (Diamanti-Kandarakis *et al* 2007).

Accumulating evidence has shown that hyperandrogenemia is highly correlated with dyslipidemia. Hyperandrogemia is associated with low HDL levels (von Eckardstein *et al* 1998) and delayed clearance of LDL (Croston *et al* 1997). Lithell *et al* (1987) also observed an inverse relationship between T concentrations and LPL activity in PCOS women. In addition to their direct contributions to dyslipidemia, androgens interfere with lipid metabolism indirectly. Studies have shown that T acts through the androgen receptor to down-regulate insulin signalling in peripheral tissues, and thus induces androgen receptor-mediated IR in PCOS (Corbould *et al* 2007, Allemand *et al* 2005).

1.6.1.3 Early Cardiovascular Dysfunction in PCOS

Several studies have focused on the markers of subclinical CVD, including early cardiovascular function and arterial morphology in PCOS (Cusson *et al* 2006). Left ventricular (LV) hypertrophy and diastolic dysfunction (measured by isovolumetric relaxation time, IVRT) are early manifestations of cardiomyopathy and predictors of cardiovascular mortality and morbidity (Levy *et al* 1991, Schannwell *et al* 2002). Women with PCOS have been found to have increased IVRT and LV mass, which are positively correlated with plasma insulin concentrations (Tiras *et al* 1999, Orio *et al* 2004a).

Arterial stiffness is associated with elevated systolic blood pressure and ventricular load, and increased arterial stiffness has been observed in PCOS women (Kelly *et al* 2002a, Lakhani *et al* 2000). In addition, endothelial dysfunction, affecting both conduit and resistance arteries is also presented in PCOS, which is an early impairment in the evolution of atherosclerosis, plaque formation and clinical CVD events (Orio *et al* 2004a, Paradisi *et al* 2001, Cussons *et al* 2006).

Women with PCOS exhibit vascular morphological changes, with increased carotid intima-media thickness (CIMT) the most common, which is an indicator of subclinical atherosclerosis (Talbott *et al* 2004). Several studies have consistently reported that CIMT or atherosclerotic index (the overall mean of the CIMT measurements at eight sites) is increased in women with PCOS (Guzick *et al* 1996, Orio *et al* 2004b). Coronary artery calcification correlates with the degree of atherosclerosis. Evidence from current studies indicates that the incidence and severity of coronary artery calcification is increased in PCOS women (Christian *et al* 2003, Talbott *et al* 2004, Cussons *et al* 2006).

1.6.1.4 Other CVD Risk Factors in PCOS

Women with PCOS also exhibit several other surrogate markers of CVD risk associated with inflammation and hemodynamics. Plasma concentrations of plasminogen activator inhibitor type 1 (PAI-1) and fibrinogen are increased in PCOS, which may increase the risk of thrombosis and atherogenesis (Atiomo et al 1998). ET-1, a potent vasoconstrictor peptide representing an early sign of abnormal vascular reactivity, is increased in both obese and non-obese women with PCOS (Diamanti-Kandarakis *et al* 2001). Kelly *et al* (2001) observed that plasma C-reactive protein (CRP) concentrations are higher in women with PCOS compared to controls. C-reactive protein is a non-specific marker of inflammation and may be an independent risk marker for cardiovascular disease (Ridker *et al* 2000). Plasma concentrations of homocysteine are also significantly increased in PCOS women (Loverro *et al* 2002), which is associated with early atherosclerosis and is an independent risk factor for cardiovascular mortality (Blacher *et al* 2002).

1.6.2 Incidence of Cardiovascular Disease Events in PCOS

In contrast to the CVD risk factors, which have been extensively assessed in PCOS, there remains limited research on CVD outcomes and events in PCOS women. Although, CVD risk factors are highly prevalent and altered vascular function is well documented, conclusive evidence that PCOS is associated with increased risk of CVD and endstage outcomes remains lacking (Wild *et al* 2000, Cussons *et al* 2006). Dahlgren and coworkers (1992) predicted that women with PCOS have a 7.4-fold relative risk for myocardial infarction, based on risk factor analysis of 33 women with polycystic ovaries compared to age-adjusted controls. However, evidence from current epidemiological studies is inconsistent due to small sample sizes, relatively short periods of follow-up, use of different diagnostic criteria and highly selected populations (Cussons *et al* 2006). The largest prospective cohort study is the Nurses' Health Study, including 82,439 women aged 20-35 years, 15% of which had irregular menstrual cycles (Solomon *et al* 2002). After a 14-year follow-up, the women with irregular menstrual cycle showed increased risk of either fatal or non-fatal coronary heart disease, and this was dependent on body mass index (BMI). Another retrospective cohort study from the United Kingdom found that women with polycystic ovaries (n=345) did not show increased mortality or morbidity from coronary heart disease compared to the age-matched controls (Wild *et al* 2000). A 10 year follow-up case-control study on Caucasian women observed that women with hyperandrogenemic chronic anovulation (n=126) were predisposed to cardiovascular events (Talbott *et al* 1995). However women with PCOS had a greater BMI than controls, and this was not corrected in statistical analyses.

In summary, PCOS is highly associated with CVD risk factors, and it is well documented that adolescent girls with PCOS demonstrate CVD risk factors, including obesity, IR and dyslipidemia (Hassan *et al* 2007). However, it is unclear if these risk factors lead to long-term CVD events and if early preventative measures can prevent the long-term development of CVD. In order to examine early development and long-term sequelae of CVD risk factors in PCOS, the use of animal models is an emergent area of research (Lakhani *et al* 2006).

1.7 Current Animal Models of PCOS

In the past decades, several animal models have been developed to determine the etiology of PCOS and to test effective treatments. To date, the most accepted animal models used are steroid-induced; however the etiology of PCOS, particularly in relation to IR remains unclear. The ideal model of PCOS should present with all the symptoms, including reproductive, metabolic and other disorders of the syndrome (Mahajan 1988a). Perhaps the best theoretical approach is for models to reflect the different phenotypes found in the general population. Due to the heterogeneous nature of PCOS, it is has been very difficult to develop animal models that mimic both the abnormal pathological and metabolic conditions of PCOS. Hence, certain considerations need to be made when using or establishing an animal model of PCOS: 1) The animal model should not only have a definitive reproductive cycle, but also well-known anatomical, biological and biochemical features, 2) The anatomical and physiological features of the model should be as similar as possible to those of human PCOS phenotypes (Azziz *et al* 2006a), and 3) the most suitable model should be chosen according to study purposes or hypotheses to be tested. The remainder of this review will focus on current animal models used to study PCOS, and will highlight models associated with the metabolic syndrome and CVD risk factors (Table 1-6).

Table 1-6. Biochemical profile and CVD related characteristics in animal models of PCOS.

Species	Method		Hormone profile	Metabolic disorders	Early CVD dysfunction
Rats	DHEA		T↑, E_2↑, P_4↑, LH↑/-, FSH ↑/-, PRL↑	insulin↑, glucose↑ resistin mRNA level of white adipose tissue↑	
	TP		T↑, E_2↓, P_4↓, LH↑, FSH↓, PRL↑	glucose/insulin↓, glucose↓/-, insulin↑	
	DHT		T-, E_2-, P_4↓	BW↑, IR, TC-, TG-, free fatty acids-, HDL - leptin level↑, body fat↑, adipocyte size↑	
	EV		T↓, E_2↓, P_4↑, LH↓, FSH↓	BW-, insulin sensitivity-	hypertension
	RU486		T↑, E_2↑, P_4↓, LH↑, FSH↓, PLR↑	BW-, IGF-I↑	aorta stiffness index↑ aorta compliance↓ BP-
	Letrozole		T↑, E_2↓, P_4↓, LH↑, FSH↑	BW-, insulin sensitivity-, TC-, TG-, free fatty acids-, HDL-	
	JCR:LA-*cp/cp*		T↑, E_2-	BW↑, IR, TG↑, TC↑, apoB48↑, HDL-, LDL-, leptin↑	intimal lesions ischemic lesions
Rhesus Monkey	Prenatally TP Treatment	Early Treatment	T↑, LH↑	BW↑, IR, IGT, FFA↑, impaired insulin secretion, leptin↑, incidence of T2D↑	
		Late Treatment	T↑, LH-	BW↑, IR, FFA-	
Ewe	Prenatally TP Treatment		T↑, LH↑, FSH-	BW-, IR, TC↑, LDL↑, HDL-, TG-	hypertension

apoB48, apolipoprotein B48; BP, blood pressure; BW, body weight; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E_2, estradiol; EV, estradiol valerate; FFA, free fatty acid; FSH, follicular stimulating hormone; HDL, high density lipoprotein; IR, insulin resistance; LDL, low density lipoprotein; LH, luteinizing hormone; P_4, progesterone; PRL, prolactin; T, testosterone; T2D, type 2 diabetes; TC, total cholesterol; TG, triglyceride; TP, testosterone propionate.

1.7.1 Rodent Models of PCOS

To date, rodents are the most widely used animal to study PCOS, benefiting from their smaller size, short lifespan, high reproduction index and different genetic strains (Singh 2005). Induction of the syndrome includes use of pharmaceutical induction, exposure to constant light and application of transgenic technology. However, research on the metabolic aspects of these models is limited, and there are still major restrictions that need to be considered when testing hypotheses in these different models.

1.7.1.1 Androgens-induced Rodent Models of PCOS

Hyperandrogenism is the primary manifestation of PCOS. One etiological hypothesis of PCOS is that exposure to excessive androgens early in life leads to PCOS in adulthood. It was reported three decades ago that elevated concentrations of circulating androgens in the rat effected ovarian follicular maturation and cyst formation (Parker *et al* 1976). Several androgens have been used to induce PCOS in rats, including dehydroepiandrosterone (DHEA), testosterone propionate (TP) and 5α-dihydrotestoterone (DHT).

1.7.1.1.1 Dehydroepiandrosterone-induced Rats

DHEA is the first androgen to rise in the female peripubertal period (Apter *et al* 1994, Mahesh et al 1962). It has been demonstrated that nearly 50% of follicular-synthesized T can be derived from circulating dehydroepiandrosterone sulfate (DHEA-S) (Haning *et al* 1994). DHEA was first used by Roy *et al* (1962) to induce PCOS in rats. Typically, prepubertal rats, aged approximately 22 days, are injected daily with DHEA (6 mg/100 g body weight, dissolved in 0.2 ml of sesame oil) for up to 20-27 days. Following treatment, rats become acyclic and anovulatory (Knudsen et al 1975a, b, Parker and Mahesh, 1976).

Ovarian Morphology: Ovaries of DHEA-induced rats become cystic with normal capsules and a decreased number of corpora lutea. Cyst size varies from 0.45-2.2mm in diameter and the granulosa cells become degenerated (Knudsen *et al* 1975a, b, Parker and Mahesh, 1976). The ovarian weight of DHEA-treated rats is increased (Wang *et al* 2004).

Endocrine Hormone Profile: Serum T, E_2, FSH, LH and prolactin concentrations, especially concentrations of DHEA, become significantly increased in the DHEA-induced rats compared to control animals (Parker and Mahesh 1976, Lee *et al* 1991, Wang *et al* 2004). Whilst no changes in plasma FSH and LH concentrations have been reported by other groups (Anderson *et al* 1997, Henmi *et al* 2001).

Cardiometabolic Abnormalities: Wang *et al* (2004) showed that fasting serum glucose and insulin concentrations were significantly increased in DHEA-induced rats. They also found the resistin mRNA level of white adipose tissue was elevated, which may mediate the development of IR. However, no other metabolic disorders of the DHEA-induced rats have been reported.

Limitations: Daily injection is invasive and may stress the animals by interfering with normal hormone and estrous cycles. No further studies have evaluated the metabolic parameters of this model.

1.7.1.1.2 Testosterone Propionate-induced Rats

Testosterone is used to induce polycystic ovaries in immature female rats (Belooseske *et al* 2004, Ota *et al* 1983). In this protocol 21-day old animals are injected daily with TP (1 mg/100 g body weight dissolved in propylene glycol) for up to 35 days (Belooseske *et al* 2004).

Ovarian Morphology: The ovaries of TP-induced rats have large multiple cystic follicles, hyperthecosis and a thickened capsule. No corpora lutea develop up to 56 days of age, in contrast to control animals which show corpora lutea at the age of 42 days. The proportion of preantral follicles increases following TP treatment (Belooseske *et al* 2004), which may be associated with increased early follicular development, as observed in human PCOS.

Endocrine Hormone Profile: Serum T, LH and prolactin levels are increased, whereas FSH, progesterone and E_2 levels are decreased in TP-treated rats (Belooseske *et al* 2004, Ota *et al* 1983).

Cardiometabolic Abnormalities: Belooseske *et al* (2004) found that in TP-treated rats fasting glucose concentrations were normal/decreased and insulin was increased, leading to a significantly reduced glucose/insulin ratio. These results have shown that excess T may lead to IR. T acts through the androgen receptor to down-regulate insulin signalling in peripheral tissues (Corbould *et al* 2007, Allemand *et al* 2005), and thus induces androgen receptor-mediated IR in PCOS.

Limitations: The TP-induced rats need daily injection of TP to induce acute polycystic ovaries condition, and few studies have been done to evaluate metabolic functions in this model.

1.7.1.1.3 5a-dihydrotestoterone -induced Rats

Most recently, Mannerås *et al* (2007) demonstrated that DHT, a non-aromatizable androgen, can induce features of both ovarian and metabolic aberrations of PCOS in rats. Three-week-old rats were implanted subcutaneously with 90-d continuous-release pellets containing 7.5mg DHT (daily dose 83ug) (Manneras *et al* 2007). Plasma DHT concentrations were 1.7-fold higher than those of control animals. After 11-13wks of the implantation, the DHT-treated rats developed acyclicity and polycystic ovaries.

Ovarian Morphology: Ovaries of DHT-treated rats display an increased incidence of atretic follicles and cysts with reduced granulosa cell layers and thickened theca interna cell layers. Interestingly, the ovarian weight of the DHT treated animals was decreased, which is not consistent with human PCOS.

Endocrine Hormone Profile: The plasma concentrations of T and E_2 were normal, whilst progesterone levels were decreased indicating anovulation (Mannerås *et al* 2007).

Cardiometabolic Abnormalities: In addition to ovarian dysfunction, the DHT-treated rats also presented with metabolic disturbances, including increased body weight associated with intra-abdominal adipose tissue, decreased insulin sensitivity and elevated plasma leptin concentrations. Despite these metabolic anomalies that are consistent with human PCOS, the DTH-induced rats showed a normal lipid profile (Mannerås *et al* 2007).

Limitations: The ovarian weight of DHT-treated rats is decreased, which is not consistent with human PCOS. Furthermore, approximately 70% of women with PCOS are dyslipidemic, therefore the DHT-treated rodent model, which is resistant to blood lipid changes may not be representative of this common phenotype of PCOS (Legro *et al* 2001).

1.7.1.2 Estrogens-induced Rodent Models of PCOS

1.7.1.2.1 Estradiol Valerate (EV)-induced Rats

Estradiol valerate is a long-acting estrogen and its administration to rats causes both hypothalamic and pituitary dysregulation of GnRH (Brawer *et al* 1978, 1986, Simard *et al* 1987, Carriere *et al* 1988). This results in improper release and storage of LH, which is the key pathogenic factor in the development of PCOS (Brawer *et al* 1978, 1986, Simard *et al* 1987, Carriere *et al* 1988). A single dose of EV (2 mg) to the young adult cyclic rats induces anovulation and polycystic ovaries within 8 weeks (Brawer *et al* 1978, 1986).

Ovarian Morphology: Ovaries of the EV-induced rats are smaller than their control counterparts, and are characterized by large cystic follicles with less granulosa cell layers and thickened theca cell layers (Brawer *et al* 1986). Ovaries from EV-induced rats also show a high incidence of atretic/degenerative secondary follicles and the ovaries become devoid of post-ovulatory corpora lutea (Brawer *et al* 1986).

Endocrine Hormone Profile: Basal plasma LH concentrations of the EV-induced rats are decreased during early stages of PCOS induction (8-10 wks after EV treatment) and they demonstrate a reduced response to LH-releasing hormone (LHRH). Twenty-two weeks after treatment, LH levels of EV-treated rats tend to increase, but still remain lower than control animals. Plasma T, E_2 and FSH concentrations are decreased and progesterone concentrations are increased in EV-induced rats (Schulster *et al* 1984, Brawer *et al* 1978, 1986, Stener-Victorin *et al* 2005).

Cardiometabolic Abnormalities: Estradiol valerate induced rats have been shown to develop hypertension, which may be related to increased sympathetic and hypothalamic-pituitary-adrenal (HPA) axis activity (Stener-Victorin *et al* 2005). However, this EV-induced model fails to develop obesity or insulin insensitivity, which are common factors in human PCOS (Stener-Victorin *et al* 2005).

Limitations: Plasma T and LH concentrations are decreased in EV-induced rats. Indeed, decreased T concentration renders this model unsuitable to study PCOS, which has been recognized as an androgen excess disorder. In addition, the EV-induced model has been likened to the multicystic ovary condition in women with hypothalamic amenorrhea, typically related to weight loss and is characterized by small ovaries containing large cystic follicles without stroma hypertrophy and decreased plasma LH concentrations (Adams *et al* 1985). Furthermore, the symptoms of EV-treated rats can be improved by opiatergic blockade, which is similar to women with this specific multicystic ovary condition (Wildt *et al* 1987, Khoury *et al* 1987, Carriere *et al* 1989). The EV-induced model does not demonstrate obesity or IR, therefore is not a suitable model to study CVD risk factors in PCOS.

1.7.1.2.2 Estradiol-induced Rats

McCarthy *et al* (1990) have found that long term exposure of female rats to physiological levels of E_2 induces PCO and might be superior to the one dose EV treatment, which causes a sudden disturbance in endocrine metabolism. Young adult cyclic female rats are given subcutaneous E_2 implants for 7 wks, resulting in vaginal cornification and acyclicity (McCarthy *et al* 1990).

Ovarian Morphology: The ovaries from E_2-induced rats are characterized by an enlarged size, increased number of atretic antral follicles and a hypertrophied stroma. No corpora lutea are present in E_2-induced rats, which indicates anovulation. Compared to EV-treated rats, the cystic follicles of E_2-induced rats are greater in number yet smaller in size with a thickened theca cell layer, which has a greater similarity to human ovary morphology in the PCOS condition (Adams *et al* 1986).

Endocrine Hormone Profile: Plasma E_2 concentrations of E_2-implanted rats are slightly higher in E_2-induced rats, compared to control animals (McCarthy *et al* 1990).

Cardiometabolic Abnormalities: No studies have been performed to evaluate the metabolic parameters in this model.

Limitations: To date, no studies have been done on androgen levels or metabolic functions in E_2-implanted rats.

1.7.1.3 Antiprogesterone-induced Rodent Model of PCOS

Antiprogesterone (RU486 or Mifepristone) is a synthetic steroid that has a high affinity for progesterone receptors and acts as a progesterone antagonist (Philibert 1984). Normally, progesterone is synthesized primarily by the corpora lutea post ovulation. Progesterone is also secreted by the adrenal gland and plays a significant role in regulating gonadotrophin secretion, follicular development, and ovulation (Buffler *et al* 1974, Caligaris *et al* 1971, Mori *et al* 1977). Progesterone inhibits the frequency of GnRH pulse. When treated with RU486, progesterone concentration is decreased in the animals as observed in human PCOS, which increases the frequency of GnRH. As discussed above, when the frequency of GnRH is increased, LH secretion predominates and the ratio of LH/FSH is increased leading to increased ovarian T secretion (Dunaif

1999, Sánchez-Criado *et al* 1990, 1993). Consequently, excess T can result in follicular atresia and anovulation (Sánchez-Criado *et al* 1990), similar to the pathophysiology described in the development of PCOS in women (Rebar 1989, Ruiz *et al* 1997). Interestingly, human studies have also shown RU486 administration during the follicular phase of the ovarian cycle causes abnormal follicular development and anovulation in healthy women (Liu *et al* 1987, Luukkainen *et al* 1988). In 4-day old cyclic rats, PCO is induced when RU486 is given subcutaneously (4mg) for 8 consecutive days beginning on the first day of estrus (Sánchez-Criado *et al* 1993, Ruiz *et al* 1996).

Ovarian Morphology: RU486 treated rats have enlarged ovaries with arrested follicular development, thickened thecal cell layers and an increased frequency of large atretic follicular cysts (>2mm), with different degrees of luteinization (refering to the hypertrophy of granulosa cells secreting progesterone) (Sánchez-Criado *et al* 1993, Ruiz *et al* 1996).

Endocrine Hormone Profile: The serum concentrations of LH, T, E_2 and prolactin are increased, whereas FSH levels are decreased in RU486 treated rats compared to control animals. The ratio of LH/FSH and T/E_2 are also increased in this model (Sánchez-Criado *et al* 1993, Ruiz *et al* 1996, 1997).

Cardiometabolic Abnormalities: A limited number of studies have reported metabolic changes and CVD risk factors in RU486 treated rats. In one study, serum IGF-I levels were found to be increased and may have a role in the ovarian abnormalities induced by RU486 treatment in the rats (Ruiz *et al* 1997). More recently, an increase in aortic stiffness index and decreased aortic compliance were observed in RU486-induced rats (Lakhani *et al* 2006), indicating that RU486 treated rats may be a good model to study premature CVD in PCOS. Lakhani *et al* (2006) also found that serum insulin concentrations tended to be elevated. However, the blood pressure and body weights of RU486 treated rats were found to be normal in this study.

Limitations: The differing hormonal environment between this rodent model and PCOS women may limit its application. For example, prolactin is the main luteotropic hormone in the rat, versus LH in humans (Smith *et al* 1975). When given RU-486, the opposing action of progesterone on E_2 disappears, leading to an enlarged pituitary and prolactin hypersecretion. Increased prolactin simulates the corpora lutea and the granulosa cells of the atretic follicles to secrete progesterone and results in the formation of luteinized cysts (van der Schoot *et al* 1987, Sommers 1980, Erickson *et al* 1993). Therefore, the ovaries of RU486-induced rats contain different degrees of luteinized cysts, similar to a multicystic condition (Clement 1987), which is considered pathologically different to the human PCOS condition.

1.7.1.4 Letrozole-induced Rodent Model of PCOS

Aromatase is the key enzyme that converts T and androstenedione into E_2 and estrone, respectively. It is widely expressed in human tissues, such as placenta, ovary, and testis (Corbin *et al* 1999). Decreased aromatase activity in the ovary is one of the pathophysiological hypotheses of PCOS development (Diamanti-Kandarakis 2008). Letrozole is a non-steroidal aromatase inhibitor, which decreases the activity of aromatase. Animals treated with letrozole have reduced conversion of androgens to estrogens, resulting in excess ovarian T production and decreased estrodiol levels (Corbin *et al* 1999). Excess T in the ovaries of the letrozole-induced rats is likely to cause polycystic ovaries directly (Sánchez-Criado *et al* 1990). Reduction of estrogen in the letrozole-induced rats weakens the negative feedback on LH production in the pituitary, causing increased LH levels (Ajika *et al* 1972), further stimulating theca cells to secrete T. Typically, six-week-old female rats (puberty) are administered letrozole orally at doses of 0.1, 0.5 and 1.0 mg/kg daily for 21 days, after which they become acyclic with histological and biochemical features of human PCOS.

Ovarian Morphology: The ovarian morphologic changes of letrozole-induced rats include the development of cysts with hyperplasia of interna theca cells and a thickened ovarian capsule. The number of corpora lutea is decreased, indicating oligo-/anovulation. However, unlike human PCOS, the ovarian weight of letrozole-induced rats remains unchanged (Kafali *et al* 2004).

Endocrine Hormone Profile: The serum T and LH levels of letrozole-induced rats are significantly elevated, and the progesterone and estradiol levels are decreased in a dose-dependent manner. However, in this model, the FSH level is markedly increased in the higher dose groups (0.5 and 1.0mg/kg), which are not the typical characteristics of human PCOS (Kafali *et al* 2004).

Cardiometabolic Abnormalities: Recently, Manneras *et al* (2007) assessed the metabolic characteristics of a letrozole-induced rodent model of PCOS, as well as its reproductive functions. They found that continuous administration of letrozole (via a subcutaneous implant) to female rats before puberty (at 3 wks of age) continuously through to adulthood (12-16 wks of age) failed to induce the metabolic disorders observed in human PCOS, including visceral obesity, decreased insulin sensitivity and dyslipidemia.

Limitations: The letrozole-induced rodent model of PCOS shows normal ovarian weight and increased FSH concentrations, which are not typical in human PCOS. Furthermore, the letrozole-induced rats do not develop metabolic disorders. Thus, the letrozole-induced rat model of PCOS does not appear to be suitable for studies of the metabolic syndrome and CVD risk in PCOS.

1.7.1.5 Constant Light-induced Rodent Model of PCOS

Spontaneous ovulation requires a gonadotropin surge that is controlled by circadian rhythms, and consequently the cycle of light-dark photoperiods can affect ovulation (Everett 1964, McCormack *et al* 1978). Exposure of female rats to constant light interferes with LH secretion and causes dysregulation of the hypothalamic secretion of

GnRH leading to the development of polycystic ovaries (Singh 1969a, b, Bredshaw *et al* 1966, Lowton *et al* 1967, Takeo *et al* 1984, Beys *et al* 1995). Studies have shown that with constant exposure to light for 7-14 wks, adult Sprague-Dawley rats become anovulatory and have atrophic ovaries.

Ovarian Morphology: The ovaries of constant-light induced rats are characterized by an increased number of tertiary follicles and/or follicular cysts, a decreased number of corpora lutea and a thickened capsule, as observed in human PCOS.

Endocrine Hormone Profile: The hormonal changes of constant-light induced rats include normal or decreased T levels, normal LH levels, elevated E_2 levels and lowered FSH and progesterone levels. (Takeo 1984, Mahajan *et al* 1988a,b). On return to normal light/dark photoperiods the normal ovarian cycling is restored. Compared to other induced PCOS models, the constant-light model is less invasive and animals develop the disorder gradually, similar to the chronic course of PCOS in women.

Cardiometabolic Abnormalities: No studies on metabolic function of this animal model have been reported.

Limitations: The constant-light induced rodent model does not develop hyperandrogemia, and LH concentration is normal in this model, which are not consistent with human PCOS.

1.7.1.6 Transgenic Rodent Models of PCOS

In addition to the induced rodent models of PCOS, transgenic animal models represent a useful tool to determine the genetic and physiological factors involved in the PCOS condition. To date, published transgenic rodent models of PCOS include transgenic mice with ovarian expression of human IGF- I (LHr-hIGF-I transgenic mice) (Dyck *et al* 2001), transgenic mice with constitutively elevated LH level (bLHβ-CTP transgenic mice) (Kero *et al* 2003), and transgenic mice constitutively expressing a stable variant of active human plasminogen activator inhibitor-1 (PAI-1) (Devin *et al* 2007). All of these transgenic mice models develop polycystic ovarian morphology and increased T concentrations similar to human PCOS. However, few studies have been performed on the metabolic aspects of the transgenic mice, and only the bLHβ-CTP transgenic mice have been reported to present with obesity and increased serum leptin and insulin concentrations (Kero *et al* 2003). Therefore, transgenic mice may be promising models of PCOS, but further studies are needed to systematically characterize the transgenic mice as models of PCOS.

In summary, the induced rodent models of PCOS discussed above, to some extent, convey morphologic and endocrine features similar to human PCOS. However, the hormonal changes cause acute polycystic ovaries condition and removal of these inducers re-establishes normal ovarian cycle in some models. These models do not necessarily reflect the chronic PCOS condition as observed in women. Furthermore, these models lack all the features of the metabolic syndrome limiting their use in the translation to understanding the human PCOS condition. Therefore, the early etiology and long term metabolic aspects and CVD risks in PCOS remain difficult to study in these models.

1.7.1.7 The JCR:LA-*cp/cp* Rodent Model of PCOS

The JCR:LA-*cp* rat is a unique strain that spontaneously expresses the *cp* gene, a stop codon in the extra-cellular domain of the leptin receptor (ObR) (Wu-Peng *et al* 1997). Animals with homozygous autosomal recessive *cp* gene (*cp/cp*) have a complete absence of the ObR, which mediates the development of obesity, IR, hyperlipidemia and CVD (Russell *et al* 1998). A long breeding history of the female JCR:LA-*cp/cp* rats has also demonstrated reproductive dysfunction. Unlike most of the other models summarized above, which need invasive daily injections or implants to induce the polycystic ovaries condition, the JCR:LA-*cp/cp* genotype appears to develop hyperandrogenemia (O'Brien *et al* 2000), infertility and abnormal ovarian morphology spontaneously (unpublished data), similar to the development of PCOS in adolescents and women (Franks 2002).

Ovarian Morphology: Preliminary observations have shown that the ovaries from the PCOS *cp/cp* animals are cystic in appearance.

Endocrine Hormone Profile: A previous study showed that the serum T concentration of PCOS *cp/cp* animals was double that of control animals, and there was no difference of E_2 concentrations between the two groups (O'Brien *et al* 2000).

Cardiometabolic Abnormalities: The female JCR:LA-*cp/cp* rats demonstrates obesity, IR and dyslipidemia (O'Brien *et al* 2000). Our preliminary studies have shown that the female JCR:LA-*cp/cp* rats have increased LV mass, reduced % ejection volume and % fractional shortening, which are associated with LV dysfunction, compared to control animals (unpublished data). Our initial vascular reactivity studies using aortic rings have also shown that the female JCR:LA-*cp/cp* rats have impaired endothelial function, characterized by impaired vasodilation in response to acetycholine (Ach), and an exacerabated relaxation response in the presence of the NO donor sodium nitroprusside (SNP) (unpublished data).

Although, mutations of the leptin receptor gene are rare in humans, the impaired leptin receptor function in the JCR:LA-*cp/cp* rat causes similar leptin resistance or hyperleptinemia observed in obese PCOS women (Mantzoros *et al* 1997). Leptin resistance is associated with increased food intake and reduced energy expenditure, leading to obesity, IGT and compensatory hyperinsulinemia (Kieffer *et al* 1996, Rohner-Jeanrenaud *et al* 1997, Goumenou *et al* 2003), which are thought to mediate the development of PCOS (Baillargeon *et al* 2007). The male JCR:LA-*cp/cp* rat is a well established model of the metabolic syndrome. Studies have shown that the male JCR:LA-*cp/cp* genotype demonstrates early vascularopathy by 3 mths of age and further develops myocardial and aortic atherosclerotic lesions by 9 mths of age. The female JCR:LA-*cp/cp* rat develops the similar but milder metabolic and cardiovascular disorders with moderate IR and delay in the development of CVD, compared to the male (O'Brien *et al* 2000).

Therefore, the female JCR: LA-*cp/cp* rat may be a putative animal model for the study of PCOS in the metabolic syndrome and associated cardiovascular disease risk. Further studies are needed to systematically characterize the JCR:LA-*cp/cp* rat as a model of PCOS.

1.7.2 Other Animal Models of PCOS

Although the rodent is widely used to generate animal models of PCOS, it does have some major dissimilarities to the human PCOS condition. Firstly, the rodent's estrous cycle is only 4-5 days and it is difficult to determine the luteal phase from the follicular phase, compared to the human menstrual cycle. Secondly, the actions of hormones in rats may be different from that in human, such as prolactin, as discussed above. Consequently, prenatally androgenized rhesus monkeys and ewes, and E_2-induced guinea pigs have been developed to study PCOS (Faiman 1988, Dumesic *et al* 1997, 2007, Goy *et al* 1988a,b, Abbott *et al* 1998, 2002a, Thornton *et al* 1983).

1.7.2.1 Prenatally Androgenized Rhesus Monkeys

It is believed that *in utero,* excess androgen may interfere with multiple organ system programming leading to a heterogeneity of adult metabolic phenotypes of PCOS (Eisner *et al* 2000, Resko *et al* 1987). To induce *in utero* androgen excess, rhesus monkeys are injected daily with 10 mg TP subcutaneously for 15–35 consecutive days starting between gestational days 40-44 (early gestational treatment) or between days 100-115 (late gestational treatment). In this model, the female fetuses have similar concentrations of T to those found in the male fetuses (Eisner *et al* 2000, Resko *et al* 1987).

Ovarian Morphology and Endocrine Hormone Profile: Fetuses that are exposed to excess T, both in early and late prenatal stages develop ovarian-derived hyperandrogenemia, anovulation and polycystic ovaries with enlarged stromal volume in adulthood (Abbott *et al* 2005). Thus this model meets the diagnostic criteria of human PCOS (Abbott *et al* 2005). In addition, increased LH concentrations are observed in early TP-treated monkeys, but not in late TP-treated monkeys (Dumesic *et al* 2002).

Cardiometabolic Abnormalities: The early and late TP-treated monkeys demonstrate metabolic disorders similar to human PCOS (Eisner *et al* 2000, 2003, Abbott 2002a, Dumesic *et al* 2007). The early-treated monkeys show abdominal obesity, impaired insulin secretion and glucose intolerance, which might be associated with pancreatic β-cell impairment. These animals also have increased circulation levels of leptin and free fatty acids. Collectively this early-treated model shows increased risk for developing type-2 diabetes. The late-treated monkeys present with decreased insulin sensitivity and increased adiposity (Abbott *et al* 2007).

Limitations: Phenotypes of prenatally androgenized monkeys, related to the gestational age at the time of androgen exposure, appear to reflect the heterogeneity of human PCOS. Therefore, prenatally androgenized female rhesus monkeys are a valuable model to study the heterogeneous etiology of human PCOS, as well as the metabolic syndrome and CVD risk factors. However, this model has its limitations, in particular the associated costs of maintaining the model, and although the menstrual cycle is comparable to the human, it takes a markedly longer period to develop the symptoms of PCOS compared to an induced or genetic phenotype as seen in rodent models.

1.7.2.2 Prenatally Androgenized Ewes

Exposure of excess androgens *in utero* in the ewe has been proposed to cause irreversible defects in gonadotrophin secretion leading to the PCO condition (Robinson *et al* 1999, 2005, Birch *et al* 2003, Unsworth *et al* 2005, Padmanabhan *et al* 2006, Forsdike *et al* 2007, King *et al* 2007). Pregnant ewes are injected intramuscularly with TP (100mg, biweekly) for 60 days between days 30-90 of an average 147-day pregnancy (King *et al* 2007). Young adults born from the androgenized ewe are acyclic and infertile with abnormal development of the ovary.

Ovarian Morphology: Ovaries of prenatally androgenized ewes are enlarged with increased stroma. Follicular development is abnormal with increased follicular recruitment and impaired follicular maturation, characterized by an increase in the number of developing follicles (i.e. primary, preantral, and antral follicles combined) and follicular cysts (with thin and disorganized granulosa cell layers), and decreased proportion of primordial follicles (Forsdike *et al* 2007, West *et al* 2001), consistent with human PCOS (Webber *et al* 2003).

Endocrine Hormone Profile: Young adults born from the androgenized ewe have increased plasma T and LH concentrations (Birch *et al* 2003, Clarke *et al* 1997). Androgen receptor expression has been found to be upregulated in ovaries of prenatally TP-treated sheep (Manikkam *et al* 2007), which may be responsible for the abnormal development of ovarian follicles.

Cardiometabolic Abnormalities: Prenatally androgenized ewes present with hyperinsulinemia and decreased insulin sensitivity in adulthood, but do maintain euglycemia in both the fasted and postprandial states (Recabarren *et al* 2005, Robinson *et al* 2005). A recent study by King and colleagues (2007) showed that fasting plasma glucose concentration of prenatal TP-treated ewes was increased. This model also demonstrates mild hypertension and a trend towards elevated plasma total and LDL cholesterol concentrations, commonly observed in human PCOS (King *et al* 2007). However, plasma TG and HDL levels, together with body weight remain normal in this model (King *et al* 2007, West *et al* 2001).

Limitations: The prenatally androgenized ewes do not demonstrate obesity or dyslipidemia. Therefore this model is not suitable to study the metabolic CVD risk factors, particularly obesity and dyslipidemia in PCOS.

1.7.2.3 Estradiol-induced Guinea Pigs

Studies have shown that exogenous E_2 may lead to increased incidence of atretic follicles and development of ovarian cysts in guinea pigs (Quandt *et al* 1993). Mature female pigs (5mths) can be implanted subcutaneously with E_2-containing silastic capsules on day 12 of the cycle (15-17days/cycle) for 48 hrs.

Ovarian Morphology: Following removal of the capsules (54 days), the E_2-induced guinea pigs show extended cycle length and enlarged ovaries with grossly discernible cysts (about 1mm), characterized by thickened, differentiated thecal layers and one to two layers of granulosa cells. In addition, the ovaries of E_2-treated pigs have an increased proportion of small atretic follicles and fewer healthy preovulatory follicles or corpora lutea. Therefore the ovarian morphology of the E_2-treated pigs is similar to that of human PCOS (Quandt *et al* 1993).

Endocrine Hormone Profile: Serum E_2 concentration of the E_2-induced guinea pigs is increased, with no changes in progesterone or androstenedione (Quandt *et al* 1993).

Cardiometabolic Abnormalities: No further metabolic studies have been reported on the E_2-treated guinea pig as a model of PCOS.

Limitations: Although the E_2-treated guinea pigs develop polycystic ovarian morphology similar to human PCOS, they do not have hyperandrogemia, which does not meet the diagnostic criteria of PCOS. Therefore, the E_2-treated guinea pig is not a suitable model to study the etiology and CVD risk factors of PCOS.

1.8 Summary

In order to further study the association between the metabolic syndrome and CVD risk in PCOS, it is deemed necessary to establish an effective animal model (Manneras *et al* 2007). It has been suggested that the ideal animal model should be the nonhuman primate, the rhesus monkey, in which the physiological aspects of the model most closely resemble human metabolism. However rodent models offer a more practical and cost-effective means to study PCOS. Among all the rodent models of PCOS, only the JCR:LA-*cp/cp* and DHT-induced rats present with both the reproductive and the metabolic disorders of PCOS. However the DHT-induced rats require an invasive treatment regime to cause the acute PCOS condition, and they do not develop dyslipidemia, a CVD risk factor observed in 70% PCOS women. Therefore, the JCR:LA-*cp/cp* rat, a spontaneous model, may be a more optimal model to study the etiology of PCOS in relation to the metabolic syndrome and CVD risk.

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2. Study Rationale

2.1 Rationale

Polycystic ovary syndrome (PCOS) is recognized globally as the most common endocrine disorder in women (Norman *et al* 2007). It is the main cause of anovulatory infertility in women, and is associated with several co-morbidities (Homburg 2008, Norman *et al* 2007). The prevalence of PCOS also appears to be increasing (Azziz *et al* 2006). Interestingly, there is a considerable heterogeneity of symptoms among PCOS women, which manifest as a combination of hyperandrogenism, menstrual disturbances, ovarian dysfunction and polycystic ovaries (Azziz *et al* 2006). In the past two decades, it has become apparent that PCOS is highly associated with features of the metabolic syndrome, including obesity, insulin resistance (IR) and dyslipidemia, which are risk factors for cardiovascular disease (CVD) (Hoffman *et al* 2008, Ehrmann *et al* 2005, Franks 1995, Dunaif 1997, Norman *et al* 2007). It is well documented that PCOS is frequently diagnosed during adolescence, and even adolescent girls with PCOS demonstrate CVD risk factors, such as obesity, IR and dyslipidemia (Hassan *et al* 2007). Early recognition and prompt treatment of PCOS in adolescents is deemed critical to the prevention of possible long-term health sequelae, such as diabetes and CVD (Norman *et al* 2007, Hassan *et al* 2007). However it remains unknown how these risk factors relate to the mechanisms of early development and long term health outcomes, particularly CVD in women with PCOS.

The etiology of PCOS remains unclear, with androgen overproduction and IR proposed to be the primary pathophysiological factors in PCOS development (Diamanti-Kandarakis 2008). Since Stein and Leventhal first reported PCOS in 1935 (Stein *et al* 1935) researchers have utilized several animal models, including rats, mice, hamsters, guinea pigs, and non-human primates to study the reproductive cycle, ovarian morphology, along with the hormonal changes associated with the pathophysiology of this condition. Of all these models, the rat is the most widely used due to its small size, short lifespan and high reproductive index. Numerous methods have been used to induce PCOS in rodent models, including treatments with androgens, estrogens, letrozole and antiprogesterone agents, and exposure to constant light (Singh 2005). However, many of these treatments are invasive, and cause an acute PCOS condition by interfering with the hypothalamic-pituitary-ovarian axis. Few models appear to develop the corresponding metabolic disturbances that are major features of PCOS. In addition, no model to date replicates all the metabolic anomalies, including obesity, IR and dyslipidemia. Furthermore, current induced rodent models of PCOS have been established based on the hypothesis that hyperandrogenemia is the primary defect that leads to ovarian dysfunction and/or IR. This hypothesis overlooks the important role of IR in the development of PCOS. Indeed, the lack of suitable animal models in this field has hampered the investigation of the pathophysiology of PCOS in the context of the metabolic syndrome, and the associated development of CVD risk.

The JCR:LA-*cp* rat is a unique strain that spontaneously expresses the *cp* gene, a stop codon in the extra-cellular domain of the leptin receptor (ObR) (Wu-Peng *et al* 1997). Animals with homozygous autosomal recessive *cp* gene (*cp/cp*) have a complete absence of the ObR, and become obese and insulin-resistant (Russell *et al* 1986, 1987, 1994). Whereas homozygous normal (+/+) or heterozygous (+/*cp*) animals are lean and metabolically normal (Russell *et al* 1986, 1987, 1994). The male JCR:LA-*cp/cp* rat is a well established model of the metabolic syndrome, and is one of the few animal models that spontaneously develops series of cardiovascular complications, including vasculopathy, intimal lesions and myocardial damage (Russell *et al* 1998, 2006, Vine *et al* 2007). The female JCR:LA-*cp/cp* rat develops similar but milder metabolic and cardiovascular disorders and moderate IR compared to the male rat (O'Brien *et al* 2000). A 20-year breeding history of the JCR:LA-*cp* strain has also shown that the female JCR:LA-*cp/cp* rat is infertile, and the ovaries from the *cp/cp* genotype are cystic/atretic in appearance. These reproductive characteristics presented in the female JCR:LA-*cp/cp* rat offer a potential role of the JCR:LA-*cp/cp* rat to be used as a model to study the metabolic and CVD risk factors associated with PCOS.

Among all the metabolic CVD risk factors, dyslipidemia is the most common. The prevalence of an abnormal lipid concentration (borderline or high) by the National Cholesterol Education Program guidelines approaches 70% in women with PCOS (Legro *et al* 2001). PCOS is classically associated with an atherogenic lipid and lipoprotein profile (Diamanti-Kandarakis *et al* 2007). However the extent and type of dyslipidemia are variable suggesting different mechanisms and pathophysiologies involved in the development of PCOS-associated dyslipidemia (Talbott *et al* 1998, Legro *et al* 2001, Conway *et al* 1992, Macut *et al* 2001). Most recently, postprandial dyslipidemia has also been assessed in PCOS (Bahceci *et al* 2007, Velazquez *et al* 2000). It is evident that dyslipidemia, particularly postprandial dyslipidemia is a prominent trait of IR, and IR is a common factor in both PCOS and the metabolic syndrome, contributing to the progression of atherosclerosis and CVD (Groot *et al* 1991, Zilversmit *et al* 1995, Twickler *et al* 2005). Given the high prevalence of an atherogenic pattern of dyslipidmia in PCOS women and in adolescent PCOS girls (Hassan *et al* 2007), understanding the pathogenic mechanisms of dyslipidemia in the PCOS condition may lead to specific therapies to prevent further development and progression of CVD.

It has long been believed that low-density lipoprotein (LDL) is the primary fraction lipoprotein involved in the development of atherosclerosis (Gustafsson *et al* 2004, Steinberg *et al* 2005). However, accumulating evidence has demonstrated that intestinally derived apolipoprotein B48 (apoB48)-containing chylomicron remnants (CMRs) are readily able to enter the arterial wall by penetrating into the subendothelium at a rate equal to or greater than that of LDL (Chung *et al* 1991, Karpe *et al* 1997, Proctor *et al* 2002). ApoB48, uniquely synthesized in the intestine, is a useful marker for intestinally-derived lipoproteins, including chylomicrons (CMs) and cholesterol-dense CMRs (Isherwood *et al* 1997). Increased fasting and postprandial apoB48 concentration/secretion has been observed in obese, insulin resistant and type 2 diabetic subjects (Couillard *et al* 2001, Mamo *et al* 2002, Allister *et al* 2006, Duez *et al* 2006), and recent evidence implicates intestinal overproduction as a causative factor in dyslipidemia (Federico *et al* 2006). The intestine is no longer regarded as just an absorptive organ but rather plays an active role in the regulation of whole body lipid metabolism, and contributes to dyslipidemia in several chronic diseases, including IR (Vine *et al* 2008). Although dyslipidemia is highly prevalent in PCOS, the underlying pathophysiology in the context of IR and hyperandrogenism is poorly understood. The role of the intestine in lipid metabolism, such as the production of CMs, has not been investigated in PCOS. Therefore, a representative animal model is needed to facilitate current studies in this area.

2.2 Aims and Hypotheses

Overall aim

The overall aim of this study was to characterize and establish the female JCR:LA-*cp/cp* rat as a model of PCOS in the metabolic syndrome, to evaluate CVD risk factors, including IR and dyslipidemia, and to determine the intestinal contribution to dyslipidemia in this model.

Hypotheses

The overall hypothesis of this study was that the female JCR:LA-*cp/cp* rodent model spontaneously presents with features of PCOS and the metabolic syndrome.

The specific hypotheses of this study were:

1. The JCR:LA-*cp/cp* rodent model of PCOS has hyperandrogenemia, a key clinical feature of PCOS.
2. The JCR:LA-*cp/cp* rodent model of PCOS demonstrates ovulatory dysfunction and abnormal ovarian follicular development.
3. The JCR:LA-*cp/cp* rodent model of PCOS develops obesity, IR and dyslipidemia.
4. The JCR:LA-*cp/cp* rodent model of PCOS presents with postprandial dyslipidemia, and the intestinal overproduction of lipoproteins contributes significantly to this dyslipidemia.

Specific Aims

To test the above hypotheses and to determine the onset and progressive development of CVD risk factors by achieving the following specific aims:

1. To establish the endocrine hormone profile of the JCR:LA-*cp/cp* rodent model of PCOS at 6wks (adolescent) and 12wks (young adult) of age by:
    i) Assessing plasma testosterone (T) and estradiol (E_2) concentrations.

2. To characterize the ovarian histology of the JCR:LA-*cp/cp* rodent model of PCOS by:
    i) Determining the cyclicity of the estrus cycle by a daily vaginal smear.
    ii) Determining the stages of the follicular development and the frequency of each stage in the ovaries of the JCR:LA-*cp/cp* rats.
    iii) Evaluating the ovarian histology.

3. To establish the biochemical features of the metabolic syndrome in the JCR:LA-*cp/cp* rodent model of PCOS, including IR and dyslipidemia at 6wks and 12wks of age by:
    i) Determining the fasting and postprandial glucose/insulin metabolism by a 'meal tolerance test (MTT)'.
    ii) Determining the fasting plasma apoB48 and lipids profile, including triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and LDL-C.

4. To assess the postprandial dyslipidemia and determine the intestinal contribution to dyslipidemia in the JCR:LA-*cp/cp* rodent model of PCOS by:
    i) Determining the plasma lipid profile and apo-B48 response following an 'oral fat challenge (OFC)'.
    ii) Evaluating the intestinal lymphatic CM production and lipid profile to determine the intestinal contribution to dyslipidemia.

2.3 Chapter Format

Results of this study are presented in two separate chapters, which are formatted as manuscripts and include:

Chapter 3: A novel model of spontaneous PCOS and associated characteristics of the metabolic syndrome in the JCR:LA-*cp/cp* rats.

Chapter 4: Postprandial dyslipidemia and intestinal overproduction of lipoproteins in a rodent model of spontaneous PCOS.

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3. A Novel Model of Spontaneous Polycystic Ovary Syndrome and the Metabolic Syndrome: the JCR:LA-*cp/cp* Rats

3.1 Introduction

In the past decades, several animal models of PCOS have been evaluated in order to describe different etiological hypotheses. The rat is the most widely used induced-PCOS model. Treatments to induce PCOS include androgens, estrogens, letrozole, antiprogesterone agents and exposure to constant light (Singh 2005). These treatments cause an acute state of polycystic ovary syndrome by interfering with the normal hypothalmic-pituitary-ovarian axis. A limited number of these models have focused on the metabolic abnormalities associated with PCOS. Furthermore, no model to date mimics all the metabolic abnormalities observed in the human PCOS condition. A recent rodent model has been reported to present both the ovarian and metabolic characteristics of PCOS using 5α-dihydrotestoterone (DHT) induction (Manneras *et al* 2007). However, the DHT-induced model demonstrates a normal lipid profile, which does not represent PCOS women, in which 70% have dyslipidemia (Talbott *et al* 1998, Legro *et al* 2001). Prenatally androgenized rhesus monkeys and ewes demonstrate both ovarian and metabolic features of PCOS, but the use of these models is inhibited by the expense of maintaining large animals, as well as the long time periods required to conduct experiments due to the extended reproductive cycle of these species. Presently, the long-term risk of developing type 2 diabetes and CVD have become major health concerns in PCOS (Franks 1995, Norman *et al* 2007). Therefore, a new model that presents both reproductive and metabolic disturbances is needed in order to investigate the etiology, and perhaps facilitate research into the prevention and treatment of PCOS. The significance of this public health concern is evident given the high incidence of PCOS, which ranges from 5-10% (Knochenhauer *et al* 1998, Homburg 2008), and the emerging prevalence of PCOS in young adolescents (Hassan *et al* 2007).

The JCR:LA-*cp* rat is a unique strain that spontaneously expresses the *cp* gene, a stop codon in the extra-cellular domain of the leptin receptor (ObR) (Wu-Peng *et al* 1997). Animals which are homozygous with the *cp/cp* genotype spontaneously develop obesity, IR and hyperlipidemia, compared to animals that are heterozygous (+/*cp*) or homozygous normal (+/+), which are lean and metabolically normal (referred to as +/?, Russell *et al* 1986, 1987, 1994). The male JCR:LA-*cp/cp* rat is a well established rodent model of the metabolic syndrome (Russell *et al* 1986, 1987, 1994, 2006, 2008, Vine *et al* 2007). In addition, the JCR:LA-*cp/cp* rodent model develops cardiovascular complications, including vasculopathy, intimal and myocardial lesions, which are unique among current models of diabetes and the metabolic syndrome. The female JCR:LA-*cp/cp* rat exhibits similar but more moderate metabolic and cardiovascular complications (O'Brien *et al* 2000). Furthermore, the female JCR:LA-*cp/cp* rat has been shown to be infertile over a 20-year breeding history of this strain. The ovaries of the *cp/cp* genotype were noted to be cystic/atretic, which suggested that the female JCR:LA-*cp/cp* rat may be a potential model to study spontaneous PCOS.

The aim of this study was to systematically characterize the female JCR:LA-*cp/cp* rat as a spontaneous model of PCOS. The evaluation of both reproductive and metabolic parameters was conducted in order to investigate the use of the female JCR:LA-*cp/cp* rat as a model of PCOS in the context of the metabolic syndrome.

3.2 Methods

Animals and Study Protocol

The female JCR:LA-*cp* strain, PCOS (*cp/cp, n* = 9) and control [+/?,or a 2:1 mix of rats heterozygous (*cp*/+) and homozygous normal (+/+), *n* = 9], were raised in our established breeding colony at the University of Alberta, as described previously (Russell *et al* 1986). The strain has been re-derived and established at Charles River Laboratories Inc. (Wilmington, MA, USA) with the designation Crl:JCR(LA)-Lepr^{cp}. Rats were weaned at 3wks of age and housed with a 12/12-h reversed light cycle to allow for study and testing during the dark phase of the rats' diurnal cycle. Rats on protocol were transferred from the isolated breeding colony areas to an individually ventilated caging environment (TechniplastTM, Exton, *PA, USA).* The animals had ad libitum access to standard laboratory rat chow (Lab diet 5001, PMI Nutrition International, Brentwood, MO, USA) and water. Animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee.

Animals were age-tracked from 6 wks (adolescent) to 12 wks (young adult) of age. At 6wks and 12wks of age, body weight, fasting plasma biochemical profile and serum hormones were determined. Postprandial insulin/glucose metabolism was assessed at 6wks and 12wks of age via a standardized 'meal tolerance test (MTT)' (Russell *et al* 1999). All blood samples were obtained using a standardized tail vein procedure (Proctor *et al* 2005). Control animals were euthanized at estrus, and ovaries were excised, weighed and fixed in neutral formalin (10%).

Reproductive Assessment

Vaginal smear

A vaginal smear was performed daily from 9wks of age for 20 days to determine the estrous cycle of each animal. The four stages of the reproductive cycle, including estrus, metestrus, diestrus and proestrus, were determined by Evans blue staining of vaginal cell smears, and microscopic analysis of the predominant cell type of the estrous cycle.

Ovarian Morphology Assessment

Ovaries were longitudinally and serially sectioned (4um) using three consecutive sections from the mid-ovary, and stained with hematoxylin and eosin. All sections were analyzed using a birefringence microscope by two persons blinded to the source of each ovary, and the histological sections were characterized and described by a veterinary pathologist blinded to animal identification. The slides were scanned and analyzed using Metamorph 6.0 (Molecular Devices, USA). The quantitative analysis of ovarian follicles and corpora lutea was assessed. The frequency of follicles at different stages of development and/or atretic follicles was counted. To evaluate the follicles accurately, the follicles were counted in each section. The stage of follicular development was determined as described by Dellmann (1987) in the following manner: follicles with one layer of flattened pre-granulosa cells were classed as primordial, and follicles with a single layer of cubical granulosa cells were primary. Secondary or preantral follicles contained more than one layer of granulosa cells without an antrum. The presence of a small antrum area was classed as early tertiary (Wright *et al* 1999, Webber *et al* 2003, Barnett *et al* 2006). Follicles with a large antral area, as well as compact and even granulosa cell layers were defined as late tertiary follicles (Fig. 3-1). Follicles were reported as normal or atretic according to the morphology. A degenerated oocyte nucleus, wrinkling of the nuclear membrane, vacuoles in the oocyte or degenerative changes in the granulosa layers, including cell shrinkage, pyknosis and karyorrhexis, were all regarded as signs of atresia (degeneration) of follicles (Hovatta *et al* 1997, Wright *et al* 1999, Webber *et al* 2003). Cystic follicles were defined as follicles that were dilated, filled with cavities of follicular fluid, and lined with a 1-4 cell thick layers of round to flattened granulosa cells.

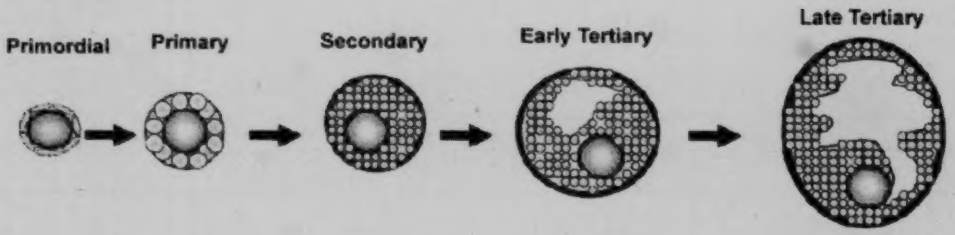

Figure 3-1. Ovarian follicular development. Adapted from Barnett *et al* 2006.

Endocrine Hormone Analysis

Total serum testosterone (T) and 17β-estradiol (E_2) concentrations were determined with commercial Enzyme-Linked ImmunoSorbent Assay (EIA) kits from ALPCO Diagnostics, USA. (T, Cat#11-TESHU-E01; E_2, Cat#11-ESTHU- E01). Assays were carried out as per the manufacturer's instructions.

Metabolic Assessment

Plasma biochemical profile

The profile of plasma biochemical characteristics following an overnight fast of animals (16h) was assessed using commercially available homogenous, enzymatic colorimetric direct and indirect assays. Triglyceride (TG) (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#998-40391/994-40491), total cholesterol (TC, WAKO, Cat#439-17501), LDL (LDL-C, WAKO Cat#993-00404/999-00504) and HDL-cholesterol (HDL-C, Diagnostic Chemical Ltd., Charlottetown, Prince Edward Island, Cat#258-20) concentrations were measured using direct colorimetric chemical enzymatic reactions. Plasma glucose was measured as per the glucose oxidase method (Diagnostic Chemicals Ltd., Cat#220-32). Insulin (ALPCO Diagnostics, USA, Cat#80-INSRT-E01) and leptin (ALPCO Diagnostics, USA, Cat# 22-LEPMS-E01v) was determined using commercially available EIA kits.

Meal tolerance test and the postprandial response

At 6wks and 12wks of age, animals were fasted overnight (16 h) and then given a 5.0 g pellet of chow as described previously (Russell *et al* 1999). Blood samples were drawn at 0, 30 and 60min following the consumption of the pellet meal using a standardized tail vein procedure. Blood was collected into tubes containing K_2EDTA and plasma was separated by centrifugation at 3000 rpm at 4°C for 10 min. Aliquots of plasma were stored at −80°C for biochemical analyses.

Analysis of the postprandial response of insulin and glucose

The postprandial response of plasma insulin and glucose from control and PCOS *cp/cp* animals was determined by the total area under the curve (AUC) using Graphpad Prizm (CA, USA), which corresponds to the total plasma concentration over the 60-minute postprandial period.

Quantitation of plasma apolipoprotein B48

The quantitation of apolipoprotein B48 (apoB48) from rodent plasma samples was done using SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), Western-blot technique coupled to ECL analysis as previously described (Vine *et al* 2007). In brief apoB48 protein was identified using a commercially available antibody to apoB (Santa Cruz Biotech, CA, Cat#sc11795). The apoB48 band was visualized using ECL (ECL-Advance, Amersham Biosciences, UK) and the imaging of proteins was conducted using a charge coupled device [CCD]-camera and Fluor-S MultiImager system (Bio-rad Laboratories, CA). The mass of apoB48 from rodent plasma was quantified using linear densitometric comparison with a known mass of the purified rodent apoB48 protein.

Statistical analysis

All results are expressed as the mean±S.E.M. Data were tested for normal distribution and differences between PCOS *cp/cp* and control groups were analysed using unpaired *t*-test with significance set at $p<0.05$. Pearson correlation analysis was performed using pair-matched values of each parameter, from each animal (Sigma Stat, Jandel Scientific, San Rafael, CA, USA).

3.3 Results

Endocrine hormone profile in the JCR:LA-cp rats

Endocrine hormone profile of the JCR:LA-*cp* rats is shown in table 3-1. At 6 wks of age, the PCOS *cp/cp* animal had higher serum E_2 concentrations compared to control animals. The serum total T concentrations tended to be increased in the PCOS *cp/cp* animals.

At 12 wks of age, the mean serum total T concentration of the PCOS *cp/cp* animals was increased significantly, 70% higher compared to control animals. The serum E_2 concentrations were similar in both groups. The T/E_2 ratio was increased in the PCOS *cp/cp* animals.

Table 3-1. Endocrine hormone profile of the JCR:LA-*cp* rats.

	6wks		12wks	
	Control (*+/?*)	PCOS (*cp/cp*)	Control (*+/?*)	PCOS (*cp/cp*)
T(ng/ml)	0.489±0.041	0.670±0.083	0.386±0.030	0.669±0.041[b]
E_2(ng/ml)	0.150±0.007	0.199±0.018[a]	0.168±0.006	0.168±0.010
T/E_2	3.297±0.227	3.918±0.696	2.335±0.208	4.200±0.130[b]

[a]$p<0.05$, [b]$p<0.001$. *t*-test performed between age-matched animals.

Reproductive assessment

Cyclicity state of the JCR:LA-cp rats

The estrus cycle of control and PCOS *cp/cp* animals is shown in Fig 3-2. All control animals had a normal estrous cycle of 3-5 days. The PCOS *cp/cp* animals showed oligo-anovulation and irregular estrous cycles compared to control animals.

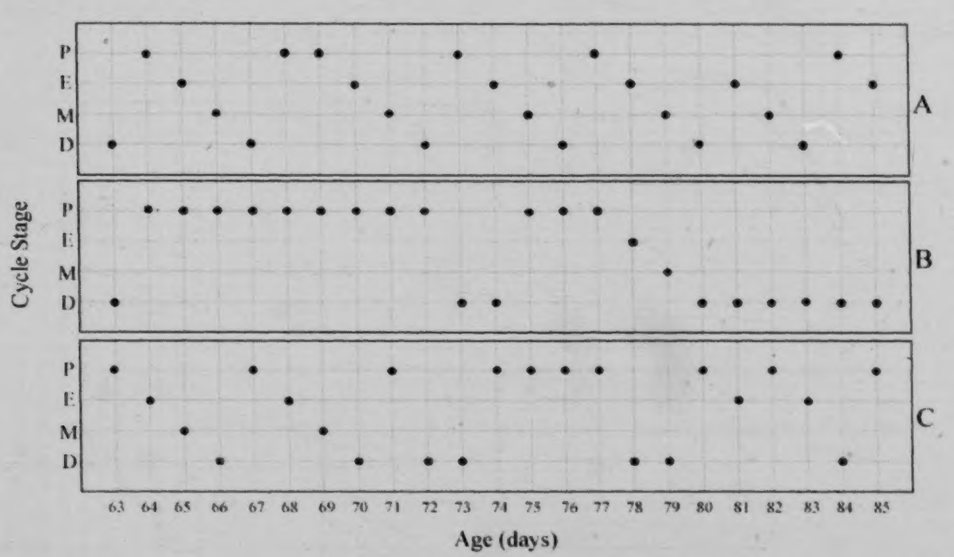

Figure 3-2. Estrous cycle patterns of a control (+/?) rat (Panel A) and two PCOS *cp/cp* rats (Panels B & C) at 63-85 day of age. The PCOS *cp/cp* rats demonstrated irregular estrous cycle with a high variability between animals. D, diestrus; E, estrus; M, metestrus; P, proestrus.

Ovarian morphology

The ovary weight was lower in the PCOS *cp/cp* animals at 12wks of age, compared to control animals (0.0394±0.0023 vs. 0.0503±0.0032, $p<0.01$). Typical changes in the ovaries of control and PCOS *cp/cp* rats are presented in Fig 3-3, 3-4, 3-5, 3-6. Interestingly, the presence of cystic follicles was observed in the ovaries of both control and PCOS *cp/cp* rats. However there appeared to be a 2-fold increase in the number of cystic follicles in PCOS *cp/cp* rats compared to control animals, but due to large variability this was not significant (Table 3-2). The majority of the cystic follicles were lined by a single layer of attenuated granulosa cells, and a fraction of these follicles were surrounded by a thin multicellular layer of round granulosa cells that appeared flattened at the cystic lumen (Figure 3-3B and 3-3C). Often these cystic follicles contained cell debris, degenerating granulosa cells and macrophages. The number of atretic follicles was significantly greater in PCOS *cp/cp* rats relative to controls ($p<0.05$). The atretic follicles were characterized by prominent apoptotic granulosa cells within the antrum and membrane, and the presence of phagocytic macrophages (Fig. 3-3A-C and 3-4A, B). The PCOS *cp/cp* animals had a tendency towards decreased corpora lutea, compared to control animals (Table 3-2). Finally, clusters of lipid droplets (Fig. 3-5) and luteinized follicles (Fig. 3-6) were commonly observed in the ovaries of PCOS *cp/cp* rats, but not in control animals.

Ovaries from the PCOS *cp/cp* animals were found to have an increased total number of follicles per section, compared to control animals (Table 3-2), and this was mainly associated with follicles in the early tertiary stage (Fig. 3-7). The PCOS *cp/cp* animals had a lower proportion of secondary follicles and an increased proportion of late tertiary follicles (Table 3-2), which may reflect a state of increased early follicular development. The mean proportion of late tertiary follicles tended to decrease in the PCOS *cp/cp* animals. The mean proportion of the atretic follicles tended to increase in the PCOS *cp/cp* animals.

Table 3-2. Analysis of follicles and corpora lutea of the JCR:LA-*cp* rats (12wks).

	Control (+/?)	PCOS (*cp/cp*)
Total follicles (/slice)	14.33±1.45	19.92±2.05[a]
Follicles at each stage (%)		
Primordial	3.40±1.86	2.84±1.28
Primary	3.84±1.90	3.17±1.61
Secondary	20.35±2.25	12.01±1.67[b]
Early tertiary	18.57±3.18	28.82±2.92[a]
Late tertiary	22.45±3.50	18.73±3.02
Atretic follicles	31.39±3.82	34.43±2.59
Cystic follicles (/slice)	0.75±0.25	1.50±0.47
Corpora lutea (/slice)	9.00±0.96	7.92±0.67

[a]$p<0.05$, [b]$p<0.01$

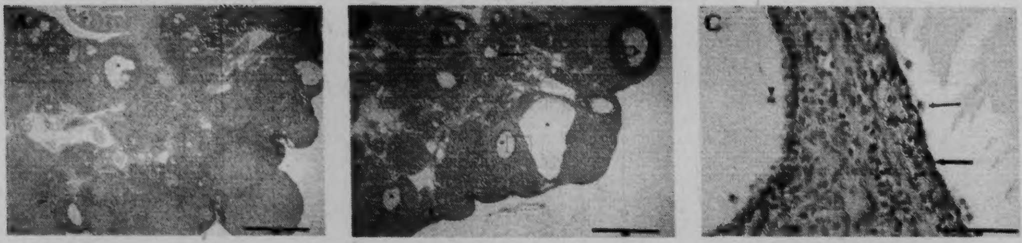

Figure 3-3. Ovarian sections from the JCR:LA-*cp* rats (12wks). A: An ovarian section from a control rat. Numerous tertiary follicles (*) are present in the cortex (Bar = 7 mm); B: An ovarian section from a PCOS *cp/cp* rat. An atretic follicle (arrow) and several cystic follicles (*) lined by either a single cell layer or thin multicellular layer of granulosa cells are shown (Bar = 7 mm); C: A higher magnification (×40) of the boxed area in Figure 3-3B. The cyst on the right is lined by a single layer of epithelioid-like cells (thick arrow) and contains a thin proteinaceous fluid, a macrophage (thin arrow) and degenerate granulosa cells. The cyst on the left is lined by a 2-4 cell thick layer of granulosa cell and contains a protein rich fluid and several sloughed degenerate granulosa cells (arrowhead, Bar = 50 μm).

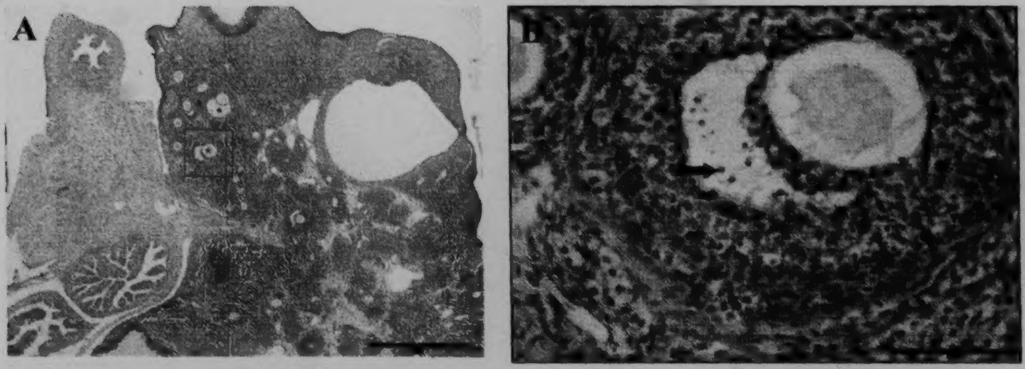

Figure 3-4. A: An ovarian section from a PCOS *cp/cp* rat (12wks). Secondary and tertiary follicles are shown, and several of these follicles are atretic (*, Bar = 7 mm). B: A higher magnification of the boxed area in Figure 3-4A. An atretic tertiary follicle characterized by periantral apoptotic granulosa cells (arrowhead) and macrophages with engulfed cellular debris (thin arrow) in the membrane granulosa, and the presence of degenerate granulosa cells in the antrum (thick arrow, Bar = 50 μm).

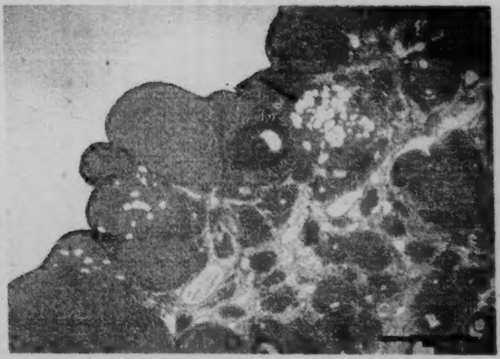

Figure 3-5. An ovarian section from a PCOS *cp/cp* rat (12wks) showing a large collection of lipid droplets (arrow) within a corpus luteum (Bar = 7 mm).

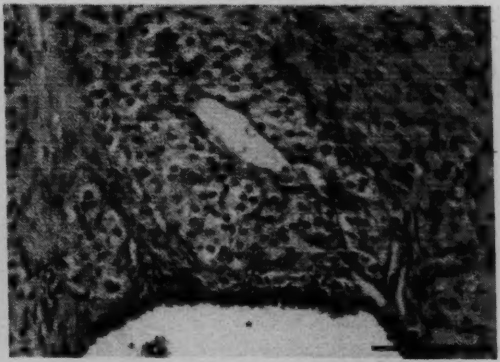

Figure 3-6. A luteinized follicle (LF) from a PCOS *cp/cp* rat (12wks). The LF is above a cystic follicle (asterisk) and is characterized by large round cells with a clear vacuolated cytoplasm and prominent central nucleus. A smaller cluster of luteinized granulosa cells is also present (thin arrow, Bar = 50 μm).

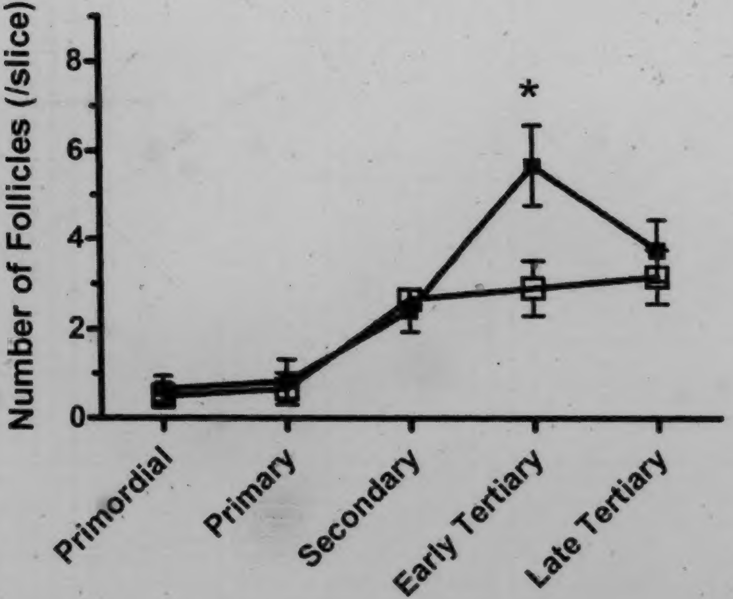

Figure 3-7. Number of normal follicles/slice at different stages in ovaries of the JCR:LA-*cp* rats (12wks). The AUC represents the total number of morphologically normal follicles/slice. The PCOS *cp/cp* rats (filled squares) had increased number of follicles, compared to controls (open squares), and the increased follicles were mainly in tertiary stage. *$p<0.01$.

Metabolic assessment

Body weight and plasma leptin concentrations

The PCOS *cp/cp* rats showed increased body weight at 6wks of age, and had a 2-fold greater increase in body weight over the 6 to 12 week period compared to control animals (182.8±9.428 vs. 63.11±8.567, $p<0.0001$). The plasma leptin concentrations were increased in the PCOS *cp/cp* animals at the age of 6wks and 12wks (Table 3-3).

Table 3-3. Metabolic and biochemical parameters of the JCR:LA-*cp* rats.

	6wks		12wks	
	Control (+/?)	PCOS(*cp/cp*)	Control (+/?)	PCOS (*cp/cp*)
Body Weight(g)	136.5±2.78	178.9±5.67[c]	201.33±5.79	367.56±10.25[c]
Leptin (ng/mL)	1.12±0.04	80.62±1.91[c]	6.50±1.35	137.80±10.13[c]
Glucose (mg/dL)	100.06±6.01	112.60±3.24	89.59±5.20	114.5±2.75[b]
Insulin (mU/L)	12.72±2.92	72.94±10.39[c]	11.67±2.22	75.46±8.60[c]
Glucose/Insulin (g/IU)	120.36±24.69	18.01±2.19[b]	119.40±20.82	18.87±3.62[b]
TG (mg/dL)	31.62±5.09	175.8±24.01[c]	22.13±3.02	904.54±94.83[c]
TC (mg/dL)	114.78±3.46	140.62±3.99[c]	86.83±4.39	147.51±13.26[c]
HDL-C (mg/dL)	43.35±3.08	50.27±5.93	35.67±2.08	41.72±4.94
LDL-C (mg/dL)	22.16±2.39	34.43±3.66[a]	19.58±2.23	16.98±2.80
ApoB48 (ug/mL)	8.77±0.61	81.86±11.27[c]	8.62±1.58	300.36±27.28[c]
TC/HDL	2.79±0.24	3.23±0.49	2.50±0.19	3.90±0.56[a]

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$. *t*-test performed between age-matched animals.

Fasting and postprandial glucose/insulin response

The fasting insulin levels of 6-wk old PCOS *cp/cp* animals were 5-fold higher than controls, and the glucose concentrations were similar between the two groups (Table 3-3). The glucose/insulin ratio, which has been proved to be a good predictor of insulin sensitivity (Legro *et al* 1998, Ducluzeau *et al* 2003), was decreased in the PCOS *cp/cp* animals. Following the MTT, the $AUC_{insulin}$ of the PCOS *cp/cp* group was significantly higher, compared to the control group ($p<0.0001$). This evidence indicates that the PCOS *cp/cp* rats develops impaired glucose/insulin metabolism early in life (at adolescence).

Whereas the AUC$_{glucose}$ did not show a significant difference between two groups (data not shown).

At 12 wks of age (adult), both the fasting plasma glucose and insulin concentrations were higher in the PCOS *cp/cp* animals (Table 3-3). The glucose/insulin ratio was decreased in the PCOS *cp/cp* animals. Following the MTT, the PCOS *cp/cp* animals exhibited the same response in insulin and glucose as at 6wks of age, with an increased insulin response to the MTT whilst maintaining euglycemia during the postprandial period (Fig. 3-8).

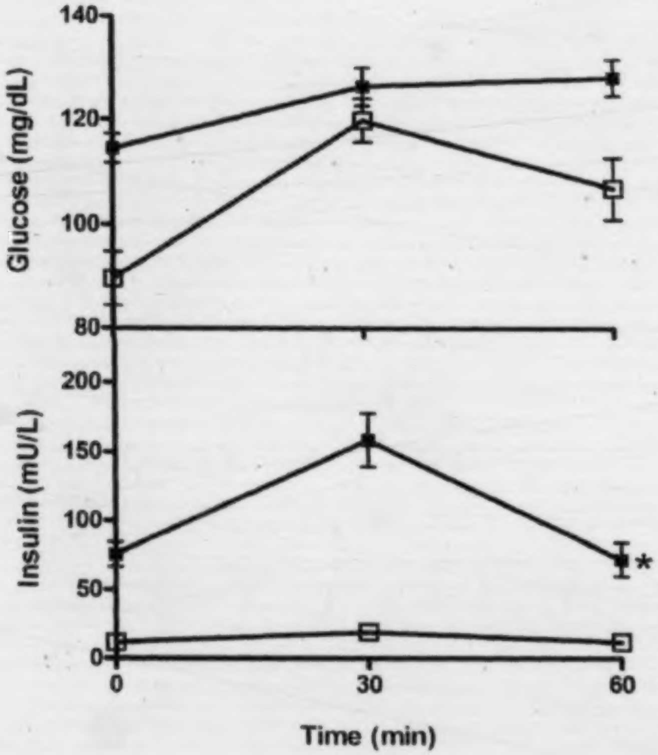

Figure 3-8. The postprandial response of plasma glucose and insulin following a MTT in the JCR:LA-*cp* rats (12wks). The PCOS *cp/cp* animals (filled squares) were shown to have a significantly greater total AUC for plasma insulin, compared to control animals (open squares) ($^*p < 0.0001$). There was no difference in total AUC for plasma glucose between the two groups.

Fasting lipid profile

The PCOS *cp/cp* animals demonstrated dyslipidemia as early as 6 wks of age, characterized by increased plasma concentrations of TG, TC, LDL-C and apoB48 (a specific marker of intestinal lipoproteins, CMs). Plasma HDL-C concentrations were similar in the two groups. At 12 wks of age, the plasma TG, TC and apoB48 concentrations of the PCOS *cp/cp* animals remained higher compared to control animals. There was no difference in plasma LDL-C or HDL-C concentrations between the two groups. The TC/HDL ratio, which has been shown to be positively associated with CVD events (Genest *et al* 2003, Ridker *et al* 2000), was also increased in the PCOS *cp/cp* animals (Table 3-3).

Correlations of fasting biochemical parameters

Correlation analysis was performed to determine the possible relationships between T and insulin, T and lipid/lipoprotein profile, as well as between fasting insulin concentrations and lipid/lipoprotein profile in the JCR:LA-*cp* rats at 12wks of age (Table 3-4). Plasma T concentrations were found to be positively and highly correlated with fasting insulin, TG, TC and apoB48 concentrations. Fasting insulin concentrations were positively correlated with fasting concentrations of TG, TC and apoB48, and the ratio of TC/HDL.

Table 3-4. Pearson correlation coefficients for fasting metabolic parameters of the JCR:LA-*cp* rats (12wks).

	BW	T	Insulin	TG	TC	ApoB48
BW	-	.793[c]	.863[c]	.837[c]	.665[c]	.930[c]
T	.793[c]	-	.672[b]	.838[c]	.490[a]	.873[c]
Insulin	.863[c]	.672[b]	-	.723[c]	.527[a]	.822[c]

Abbreviations: BW, body weight; T, testosterone; TG, plasma triglyceride; TC, total cholesterol; apoB48, apolipoprotein B48; (–), not calculated.

[a] $p < 0.05$.

[b] $p < 0.01$.

[c] $p < 0.001$.

3.4 Discussion

The aim of this study was to characterize the female JCR:LA-*cp/cp* rat as a spontaneous model of PCOS in the context of the metabolic syndrome. The female JCR:LA-*cp/cp* rats presented with both reproductive and metabolic disturbances similar to symptoms observed in human PCOS. Unlike other models that use invasive subcutaneous implants or daily injections to induce the acute polycystic ovarian condition, the JCR: LA-*cp/cp* genotype develops oligo-ovulation, hyperandrogenemia and cystic ovaries spontaneously and gradually, similarly to the chronic etiological development of PCOS in women (Franks 2002).

Reproductive assessment

The PCOS *cp/cp* animals developed hyperandrogenemia, oligo-ovulation, irregular estrous cycles, and cystic ovaries. However, compared to other induced rodent models of PCOS, the severity and degree of the latter symptoms was mild (Manneras *et al* 2007, McCarthy *et al* 1990). The moderate symptoms of PCOS in the JCR:LA-*cp/cp* rat may render it a similar model to human PCOS, which is dependent on classification, in particular hyperandrogenemia and oligo-/anovulation.

The ovaries from the PCOS *cp/cp* rats showed an increased number of atretic follicles and follicular cysts of varying size, and exhibited a significant decrease in granulosa cell layers compared to control animals. The number of corpora lutea tended to be lower in the PCOS *cp/cp* rats, indicating oligo-ovulation, which was consistent with results from the daily vaginal cytology. We also observed clusters of stromal lipid droplets in the ovaries from the PCOS *cp/cp* animals, which have not been described in the human PCOS condition, or other animal models (Franks 1995, Jonard *et al* 2004, Balen *et al* 2003, Fulghesu *et al* 2007), and may be associated with ovarian dysfunction in this model.

The follicular development in PCOS is abnormal, and evidence has suggested that this follicular mal-development may be associated with two factors: an increase in follicular recruitment and an arrest in follicular development at the small antral stage (early tertiary) (Jonard *et al* 2004). Consistently, the analysis of ovarian follicular development in this study demonstrated that the number of total follicles was increased in the PCOS *cp/cp* animals, as observed in human studies (Hughesdon *et al* 1982, Webber *et al* 2003). In addition, the increased number of follicles was observed to be mainly in the early tertiary stage, implying that the rate of early follicular development was increased and became arrested at the early tertiary stage. Although the number of atretic follicles was increased in the PCOS *cp/cp* animals, the proportion of atretic follicles was similar in the two groups. These observations are consistent with Webber's study (2003), in which the density of follicles was increased in ovaries from PCOS women, whilst the proportion of atretic follicles in polycystic ovaries from PCOS women did not differ from that in normal ovaries. Altered follicular development has been attributed to the abnormal endocrine environment, particularly androgen excess (Norman *et al* 2007). A certain amount of intra-ovarian androgens are necessary for early follicular development from primary follicles to the stage of pre-antral and small antral follicles. However, excess androgens leads to increased follicular recruitment, poor follicle maturation (follicular arrest), follicular atresia and formation of follicular cysts (Jonard *et al* 2004, Homburg 2008).

Although large cystic follicles were observed in the PCOS *cp/cp* animals, most cysts were small, and the number of cystic follicles was less than seen in other rodent models of PCOS (Manneras *et al* 2007, McCarthy *et al* 1990). Increased androgen levels are strongly related and cause the development of atretic/cystic follicles in animal models. However, unlike other animal models, the JCR:LA-*cp/cp* rat develops the PCOS condition gradually, and does not show hyperandrogemia until 12 wks of age or early adulthood. Ota *et al* (1983) performed a study to observe the process of polycystic ovary formation in the rat treated with testosterone propionate (TP), and found that the development of polycystic ovary and hyperthecosis occurred 40 days after a single injection of TP. In addition, most induced rodent models of PCOS are administered androgens, including dehydroepiandrosterone (DHEA) and dihydrotestosterone (DHT) at early time points, for example 3wks of age (Anderson *et al* 1997, Manneras *et al* 2007). Since large cysts develop gradually under the influence of excess androgens, the PCOS *cp/cp* animals may develop a greater severity of ovarian dysfunction and an increased prevalence of cystic follicles as they age and this is being further investigated.

Follicular development in PCOS women is also influenced by gonadotropin (LH/FSH) levels via the hypothalamic-pituitary-ovarian axis, which are linked to androgen levels. LH regulates the androgenic synthesis of theca cells, and FSH is responsible for regulating the aromatase activity of granulosa cells, regulating synthesis of estrogens from androgens (Balen 2004). When the ratio of LH/FSH is increased, the ovaries preferentially synthesize androgens. The production and secretion of pituitary LH and FSH are partly stimulated by the hypothalamic gonadotropin-releasing hormone (GnRH), and when the GnRH pulsatility is rapid, LH secretion predominates and the ratio of LH/FSH is increased (Haisenleder *et al* 1991), as observed in PCOS. However, the activity of the hypothalamic-pituitary-ovarian axis has not been further characterized in the female JCR:LA-*cp/cp* rats.

In this study, we have shown that the PCOS *cp/cp* rats are oligo-ovulatory using the daily vaginal cytology method, however whether these animals are totally anovulatory, that is they do not go into the estrus phase of the cycle is unknown and probably varies between animals. The 20-year breeding history suggested thse animals are infertile but further testing could be done to fully elucidate the type of infertility the *cp/cp* genptype has. Fertilization is affected by several factors, including ability to copulate, quality of the oocytes and the normal hypothalamic-pituitary-ovarian axis of endocrine feedback. We know these animals have an abnormal endocrine profile but the exact dysfunction of the hypothalamic-pituitary-ovarian axis and oocyte quality remain to be determined. In addition a copulation plug test would be helpful to further determine if these animals can indeed mate which could be the primary cause of infertility observed in this model.

Metabolic abnormalities

Obesity and leptin in PCOS

The JCR:LA-*cp/cp* rats showed increased body weight from the age of 6wks compared to control animals, which is associated with their leptin receptor defect. The prevalence of obesity (BMI≥30) in PCOS has been reported to range from 38% to 70%, which is higher compared to women in the general population and is varied by genetic and geographic factors (Hoeger *et al* 2006). Obesity has a substantial effect on the manifestation of PCOS, and is known to exacerbate IR. It is also evident that obesity is associated with a greater severity of PCOS symptoms, and that weight loss is highly effective at improving not only metabolic parameters, but also reproductive factors in PCOS, including hyperandrogemia, ovulation and fertility (Kiddy *et al* 1992, Holte *et al* 1995).

The JCR:LA-*cp/cp* rodent model has a leptin receptor defect mediating the development of obesity, IR, hyperlipidemia and CVD dysfunction (Russel *et al* 1998). Although, mutations of the leptin receptor gene are rare in humans, the impaired leptin receptor function in the PCOS *cp/cp* model causes a similar leptin resistance or hyperleptinemia as observed in obese PCOS women (Mantzoros 1997). The direct mechanistic role of increased leptin in the development of PCOS remains uncertain. Although most studies on leptin in PCOS indicate a close relationship between leptin levels and the percentage body fat, there are no reported significant differences in leptin concentrations between ovulatory and anovulatory women with PCOS (Maffei *et al* 1995, Pirwany *et al* 2001). Therefore, despite leptin concentrations being associated with body fat, they do not appear to be directly related to ovulatory function (Pirwany *et al* 2001).

Insulin resistance in PCOS

Insulin resistance has been identified as a central pathogenic factor, interfering with ovarian T synthesis and ovarian function, and as such is regarded as the link between PCOS and the metabolic syndrome (Poretsky 1999, Dunaif *et al* 1988, Baillargeon *et al* 2004, 2006, Hoffman *et al* 2008). IR has also been shown to be associated with endothelial dysfunction and increased cardiovascular risk (Petire *et al* 1996; Baron *et al* 2002). Although it is associated with IR, lean women with PCOS also demonstrate IR, suggesting the integral role of insulin in PCOS development (Dunaif 1997). Indeed approximately 80% of obese women and 30–40% of lean women with PCOS have IR (Dunaif 1997), and screening for impaired glucose tolerance in all women with PCOS has now been recommended by the task force of the Androgen Excess Society (Salley et al 2007). In this study, the PCOS *cp/cp* rats exhibited increased fasting insulin concentrations, and an increase in postprandial insulin response, which is consistent with human studies of insulin metabolism in PCOS (O'Meara *et al* 1993, Bahceci *et al* 2006). A reduced fasting glucose/insulin ratio is a useful marker of insulin sensitivity in PCOS women (Legro *et al* 1998, Ducluzeau *et al* 2003), and this was also shown to be decreased in the PCOS *cp/cp* animals.

Dyslipidemia in PCOS

Dyslipidemia is estimated to affect up to 70% of women with PCOS (Legro *et al* 2001). It has been suggested that fasting lipid levels should be routinely evaluated at the time of diagnosis of PCOS (Trivax *et al* 2007). Several studies have shown the prevalence of fasting dyslipidemia in PCOS, and most studies have shown that PCOS is associated with an atherogenic lipid profile characterized by elevated TG, total and LDL-cholesterol, lowerd HDL cholesterol (Talbott *et al* 1995, Conway *et al* 1992), and increased total apolipoprotein B (apoB) concentrations (Macut *et al* 2001). In the present study, the PCOS *cp/cp* rodent model exhibited severe fasting dyslipidemia, specifically hypertriglyceridemia, increased plasma TC and apoB48 concentrations. Indeed, hypertriglyceridemia is an independent risk factor for CVD (Assmann *et al* 1996, Hokanson *et al* 1996, Jeppesen *et al* 1998), and apoB48, a specific marker for intestinal chylomicrons (Mamo *et al* 2001), contributes to atherogenesis and CVD (Twickler *et al* 2005, Proctor *et al* 2002). Plasma HDL and LDL concentrations were found to be normal in this model, however the TC/HDL-C ratio, a sensitive and specific index of CVD risk (Genest *et al* 2003, Ridker *et al* 2000) was increased in the PCOS *cp/cp* animals.

The pathogenesis of dyslipidemia in PCOS remains controversial due to the clustering of interrelated risk factors involved in PCOS and there are discrepancies between reports on the types and severity of lipid aberrations in PCOS (Wild *et al* 1985; Talbott *et al* 1995; Rajkhowa *et al* 1997). The JCR:LA-*cp/cp* rodent model may represent one type of dyslipidemia in PCOS. Obesity, hyperandrogenemia and IR may all contribute to the development of dyslipidemia in PCOS (Diamanti-Kandarakis *et al* 2007). In this study, we observed that body weight, T and insulin concentrations were positively correlated with fasting TG, TC and apoB48 concentrations the JCR:LA-*cp* rats. Since hyperandrogemia, hyperinsulinemia and obesity are intimately interrelated, and each of these factors has independent and synergistic effects on dyslipidemia, it is difficult to determine the exact cause and pathophysiology of dyslipidemia in PCOS (Diamanti-Kandarakis *et al* 2007). However, the correlations demonstrated in this study provide possible pathways to explore in studies examining the onset of dyslipidemia in PCOS.

Hyperinsulinemia and hyperandrogenemia in the JCR:LA-cp/cp rats

Insulin and androgens have been regarded as the two main factors involved in PCOS (Franks 1995, Dunaif 1997, Tsilchorozidou *et al* 2004). In this study, we found fasting insulin and T concentrations were positively correlated in the JCR:LA-*cp* rats, which is consistent with previous human studies (Burghen *et al* 1980, Lobo *et al* 1983).

It has long been debated whether hyperinsulinemia or hyperandrogemia is the primary defect in PCOS. Several lines of evidence suggest that hyperinsulinemia may be the main culprit leading to hyperandrogemia and the manifestations PCOS (Tsilchorozidou *et al* 2004). The severity of hyperinsulinemia has been associated with the adversity of the clinical symptoms in PCOS (Conway *et al* 1992, Robinson *et al* 1993). Indeed, insulin-sensitizing treatment can decrease androgen levels, especially T levels, and also improve metabolic and endocrine symptoms, ovulatory function, menstrual cyclicity and fertility rates, independent of weight loss (Baillargeon *et al* 2004, 2006, Gambineri *et al* 2004, Azziz *et al* 2001, Pasquali *et al* 1989). Baillargeon and coworkers (2004, 2006) have observed that the insulin-sensitizer, including metformin and rosiglitazone can improve the symptoms of PCOS (increasing ovulatory frequency and ameliorating hyperandrogenemia), even in non-obese women with PCOS who demonstrated normal clinical indices of insulin sensitivity. In contrast, effects of anti-androgen treatments, including bilateral oophorectomy, GnRH-agonists, spironolactone and flutamide on IR are contradictory, with partial reversal of peripheral IR associated with the hyperandrogenism (Moghetti *et al* 1996) versus no effect on IR in women with PCOS (Diamanti-Kandarakis *et al* 1995, Nagamani *et al* 1986, Geffner *et al* 1986, Dunaif *et al* 1990).

We have shown the PCOS *cp/cp* rats demonstrate hyperinsulinemia and IR at 6wks of age and increased T concentrations at 12wks of age, which may suggest that hyperinsulinemia is the primary defect leading to PCOS in this model. For this model we propose that, leptin resistance and compensatory hyperleptinemia are associated with increased food intake, reduced energy expenditure leading to obesity and IR/hyperinsulinemia (Kieffer *et al* 1996, Rohner-Jeanrenaud *et al* 1997, Goumenou *et al* 2003), which consequently leads to hyperandrogenemia, ovulatory dysfunction and ovarian morphological changes. Therefore, unlike other current animal models of PCOS that are primarily androgen-induced, the JCR:LA-*cp/cp* rat provides a potential model of spontaneous PCOS in the metabolic syndrome.

PCOS in adolescents

It has been recognized that most adult women with PCOS show symptoms from adolescence, including irregular menstrual cycles, signs of hyperandrogenism and commonly are obese (Hassan *et al* 2007). Furthermore, adolescents with PCOS often exhibit metabolic abnormalities, such as impaired glucose tolerance (30% of incidence), IR and dyslipidemia (Palmert *et al* 2002, Hassan *et al* 2007). In this study, we observed that at 6wks of age, the PCOS *cp/cp* animals tended to have higher plasma total T concentrations compared to control animals. The 6-week old PCOS *cp/cp* animals also demonstrated IR accompanied by hyperinsulinemia, obesity and dyslipidemia, similar to the symptoms of PCOS in adolescents (Hassan *et al* 2007). Thus, our results indicate that the female JCR:LA-*cp/cp* rat may be a good model to study the early development of CVD risk factors in PCOS.

3.5 Conclusions

The results of present study support the hypothesis that the JCR:LA-*cp/cp* female rat, which spontaneously presents ovarian dysfunction, may be a useful model to study the etiology and pathogenesis of PCOS in the context of the metabolic syndrome. In addition, this model has a potential use in determining early and long-term cardiometabolic risks and possible preventative strategies targeted at the development of CVD.

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4. Postprandial Dyslipidemia and Intestinal Overproduction of Lipoproteins in a Rodent Model of Polycystic Ovary Syndrome and the Metabolic Syndrome

4.1 Introduction

Dyslipidemia appears to be the most common metabolic disturbance in PCOS, occurring in up to 70% of PCOS women (Talbott *et al* 1998, Legro *et al* 2001). Most studies examining fasting dyslipidemia in PCOS have shown that this syndrome is associated with an atherogenic lipid and lipoprotein profile, characterized by elevated triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC), lowered high-density lipoprotein cholesterol (HDL-C) and increased total apolipoprotein B (apoB) (Talbott *et al* 1995, Conway *et al* 1992, Macut *et al* 2001). However, results from these studies have shown differing profiles of dyslipidemia in PCOS, which possibly reflects the heterogeneity of the metabolic disturbances, particularly associated with IR in PCOS (Homburg 2008). Bahceci and coworkers (2007) have recently reported postprandial lipemia in PCOS women, with an elevated TG and total apoB response following an oral fat tolerance test, compared to BMI-matched controls. Postprandial lipid and lipoprotein metabolism is a dynamic process which takes place following food ingestion. Indeed, people are in a fed state for most of the day. This is important from an etiological perspective because the artery wall is exposed to postprandial plasma lipid levels rather than fasting levels (Cohn 2006). It has been hypothesized that atherogenesis is a postprandial phenomenon (Zilversmit *et al* 1979), and evidence has accumulated to demonstrate that postprandial lipemia is a major determinant of circulating lipoprotein concentrations and of cardiovascular risk (Zilversmit 1995, Karpe *et al* 1995). Meyer *et al* (1996) has shown that patients with coronary artery disease and normal fasting lipid levels have an increased TG response associated with triglyceride-rich lipoprotein (TRL, Sf>1000) fraction, and a greater total and incremental apolipoprotein B48 (apoB48) response following a fat load than patients without coronary artery disease. It is evident that dyslipidemia, particularly postprandial lipemia, is associated with IR a key feature found in both PCOS and the metabolic syndrome, and is related to premature atherosclerosis (Groot *et al* 1991, Zilversmit *et al* 1995, Twickler *et al* 2005, Baillargeon *et al* 2006).

Accumulating evidence has shown that intestinally derived chylomicrons (CMs), particularly chylomicron remnants (CMRs) are highly atherogenic particles (Proctor *et al* 2002). CMRs can permeate the arterial tissue to deliver cholesterol and initiate macrophage foam cell formation, a hallmark feature of early atherogenesis (Chung *et al* 1991, Karpe *et al* 1997). ApoB48 is specifically synthesized in the intestine and is necessary for CM assembly, therefore is a marker for the quantification of intestinally-derived lipoproteins (Kane *et al* 1983, Isherwood *et al* 1997, Federico *et al* 2006). CMs are TG-rich particles secreted by the enterocyte, and deliver dietary and endogenously synthesized lipids to the circulation via the lymphatic system. Once in circulation, CMs are hydrolyzed to produce cholesterol-dense CMRs (van Beek *et al* 1998, Redgrave 1983). In recent years, the intestine has been recognized as an active organ that is involved in the regulation of the lipid absorption, intracellular transport and secretion, and consequently affects the whole body's lipoprotein metabolism. Thus the intestine is proposed to contribute significantly to dyslipidemia in several chronic diseases, including obesity, diabetes and CVD (Vine *et al* 2008). Furthermore, current evidence also suggests that IR may affect the intestinal regulation of lipid metabolism (Federico *et al* 2006, Allister *et al* 2006, Vine *et al* 2008, Adeli *et al* 2008). Although dyslipidemia is highly prevalent in PCOS, the underlying pathophysiology in relation to the metabolic syndrome and androgen disturbances in PCOS is poorly understood. The regulation of the intestinal lipid metabolism has not been investigated in PCOS, but may be a significant contributor to dyslipidemia based on recent observations of postprandial dyslipidemia in PCOS (Bahceci *et al* 2007).

Although there are presently several rodent models of PCOS, only the DHT-induced model presents with metabolic disorders, including obesity and IR, as observed in human PCOS (Manneras *et al* 2007). However the DHT-induced model fails to demonstrate dyslipidemia, which is very common in PCOS women. In fact, the lack of suitable animal models has retarded investigations into the mechanisms involved in the development of metabolic abnormalities, such as dyslipidemia in PCOS. We have recently characterized the female JCR:LA-*cp/cp* rat as a model of PCOS in the context of obesity and IR. Unlike other animal models of PCOS that require steroid-induction, this model spontaneously develops hyperandrogenemia, oligo-ovulation, and has atretic-cystic type ovarian follicular malformation (Chapter 3). The aim of this study was to assess the postprandial lipemic response and to determine intestinal CM production in the JCR:LA-*cp/cp* rodent model of PCOS and the metabolic syndrome.

4.2 Methods

Animals and Study Protocol

Twelve-week old female rats of the JCR:LA-*cp* strain, PCOS (*cp/cp*, n=9) and control (+/?, n=9), were raised in our established breeding colony at the University of Alberta, as described previously (Russell 1995). The strain has been re-derived and established at Charles River Laboratories Inc. (Wilmington, MA, USA) with the designation Crl:JCR(LA)-Lepr^{cp}. Rats were weaned at 3 wks of age and housed with a 12/12-h reversed light cycle to allow for study and testing during the dark phase of the rats' diurnal cycle. At 12 wks of age rats were transferred from the isolated breeding colony areas to an individually ventilated caging environment (TechniplastTM, Exton, PA, USA). The animals had ad libitum access to standard laboratory rat chow (Lab diet 5001, PMI Nutrition International, Brentwood, MO, USA) and water. Animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee.

We have previously established the female JCR:LA-*cp/cp* rat as a potential model of PCOS in the context of obesity and the metabolic syndrome, as described in chapter 3. The typical ovarian morphology from PCOS *cp/cp* genotype with abnormal follicular development and cystic follicles is shown in Fig. 3-3, 3-4. These animals also display a 70% increase in serum testosterone (T) concentration, and have increased leptin concentration due to a leptin receptor defect, compared to control animals (as shown in Table 4-1).

Oral Fat Challenge and the Postprandial Response

Animals were fasted overnight (16 h) and then given a 5.0 g pellet containing 30% (w/w) lipid. Blood samples were obtained using a standardized tail vein procedure (Proctor *et al* 2005) at times 0, 2, 4, 5, 6, 8 and 10 h following consumption of the pellet meal. Blood was collected into tubes containing K_2EDTA (ethylene diamine tetraacetic acid, BD Franklin Lakes NJ USA, Cat#367835), and plasma was separated by centrifugation at 3000 rpm at 4°C for 10 min. Aliquots of plasma were stored at −80°C for biochemical analyses.

Plasma and Intestinal Lymph Biochemical Profile Assessment

The profile of biochemical parameters in plasma and lymph from control and PCOS *cp/cp* groups of the JCR:LA-*cp* strain were assessed using commercially available homogenous, enzymatic colorimetric direct and indirect assays. Total serum T concentrations were determined with commercial Enzyme-Linked ImmunoSorbent Assay

(EIA) kits from ALPCO Diagnostics, USA. (T, Cat#11-TESHU-E01). TG (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#998-40391/994-40491), TC (WAKO, Cat#439-17501), LDL-C (WAKO Cat#993-00404/999-00504) and HDL-C (Diagnostic Chemical Ltd., Charlottetown, Prince Edward Island, Cat#258-20) concentrations were measured using direct colorimetric chemical enzymatic reactions. Plasma glucose was measured as per the glucose oxidase method (Diagnostic Chemicals Ltd., Cat#220-32). Insulin was determined using commercially available EIA kits (ALPCO Diagnostics, USA, Cat#80-INSRT-E01).

Quantitation of Plasma and Intestinal Lymph Apolipoprotein B48

The quantification of apoB48 from rodent plasma and lymph samples was done using established SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), Western-blot techniques coupled to ECL analysis (Vine *et al* 2007). ApoB48 protein was identified using a commercially available antibody to apo B (Santa Cruz Biotech, CA, Cat#sc11795). The apoB48 band was visualized using ECL (ECL-Advance, Amersham Biosciences, UK) and the imaging of proteins was conducted using a charge coupled device [CCD]-camera and Fluor-S MultiImager system (Bio-rad Laboratories, CA). The mass of apo-B48 from rodent plasma was quantified using linear densitometric comparison with a known mass of the purified rodent apoB48 protein (Vine *et al* 2007).

Analysis of the Postprandial Response

The postprandial response of plasma apoB48, TG and insulin from control and PCOS *cp/cp* animals was determined by the total area under the curve (AUC) using Graphpad Prism (CA, USA). The AUC corresponds to the total plasma concentration over the 10-h postprandial period. Fasting concentration of each parameter was further subtracted from the total AUC for the postprandial period to yield the incremental area under the curve (iAUC). Importantly, the iAUC represents the change in the postprandial response (compensating for the initial concentration of each parameter measured during the fasting state). The plasma content of both apoB48 and TG during the postprandial phase is primarily due to the secretion of intestinal CMs transporting dietary lipid, therefore changes in iAUC values for apoB48 and TG provide an accurate representation of lipoprotein and lipid contribution from the intestine.

Surgical Procedure of Lymph Cannulation

The surgical procedure for lymph cannulation and isolation of intestinal CMs, was done as described previously (Vine *et al* 2002). A food equivalent pre-surgical regime to minimize the confounder of hyperphagia in the PCOS *cp/cp* rats was used. In brief, all rats (control and PCOS *cp/cp*) were fasted 48hrs prior to the surgery and then re-fed a standard 21 gm pellet 16hrs before the surgical procedure. Rats were anaesthetized (3% Isoflurane), and a mesenteric lymph duct and gastric cannula were inserted. Rats were given a constant gastric infusion of saline at 1.5 ml/hr for 5 hrs. Lymph samples were collected in EDTA (4mM), spun at 3000 for 15 min, flushed with N_2, and frozen at -80°C and the remainder was stored in 4°C (for particle size and lipid profile analysis). Intralipid (Kabi Pharmacia, Sweden) 2% (v:v) was infused at 1.5 ml/hr for a further 5 hrs and lymph was collected.

Statistical Analysis

All results are expressed as the mean±S.E.M. Data were tested for normal distribution, and differences between PCOS *cp/cp* and control groups were analyzed using unpaired *t*-test with significance set at $p<0.05$. Pearson correlation analysis was performed using pair-matched values of each parameter from each animal (Sigma Stat, Jandel Scientific, San Rafael, CA, USA).

4.3 Results

Fasting Biochemical Profile

The PCOS *cp/cp* animals were hyperinsulinemic with fasting insulin concentrations eight-fold higher than those of the control animals (Table 4-1). Fasting plasma concentrations of TG and TC were increased significantly in the PCOS *cp/cp* animals. The apoB48 concentrations (intestinally derived lipoproteins) were 34 fold higher in the PCOS *cp/cp* animals. However, there was no difference in HDL-C or LDL-C concentrations between two groups, which are commonly described as significant CVD risk factors (Table 4-1).

Table 4-1. Fasting plasma biochemical parameters in the JCR:LA-*cp* rats.

	Control (+/?)	PCOS (*cp/cp*)
Body Weight(g)	201.33±5.79	367.56±10.25^{b}
T(ng/ml)	0.39±0.03	0.67±0.04^{b}
Leptin (ng/mL)	6.50±1.35	137.80±10.13^{b}
Insulin (mU/L)	9.05±1.18	86.14±9.54^{b}
TG (mg/dL)	22.13±3.02	904.54±94.83^{b}
TC (mg/dL)	86.83±4.39	147.51±13.26^{b}
HDL-C (mg/dL)	35.67±2.08	41.72±4.94
LDL-C (mg/dL)	19.58±2.23	16.98±2.80
ApoB48 (ug/mL)	8.62±1.58	300.36±27.28^{b}
TG/apoB48	33.11±5.16	32.75±3.94

^{a} $p<0.05$, ^{b} $p<0.001$

Further analysis found that fasting insulin concentrations had a strong correlation with T concentrations. Circulating T concentrations were shown to be correlated with fasting plasma TG and apoB48 concentrations, consistent with previous results in Chapter 3 (See Table 4-3).

Postprandial response of TG, apoB48 and insulin

The postprandial response of TG and apoB48, as measured by total AUC and incremental AUC (iAUC) was increased significantly in PCOS *cp/cp* animals (Fig. 4-1, 4-2, Table 4-2).

The postprandial response of insulin in PCOS *cp/cp* animals, measured by AUC and iAUC was significantly increased (15-fold higher and 26-fold higher, respectively), compared to control animals (Fig. 4-3, Table 4-2).

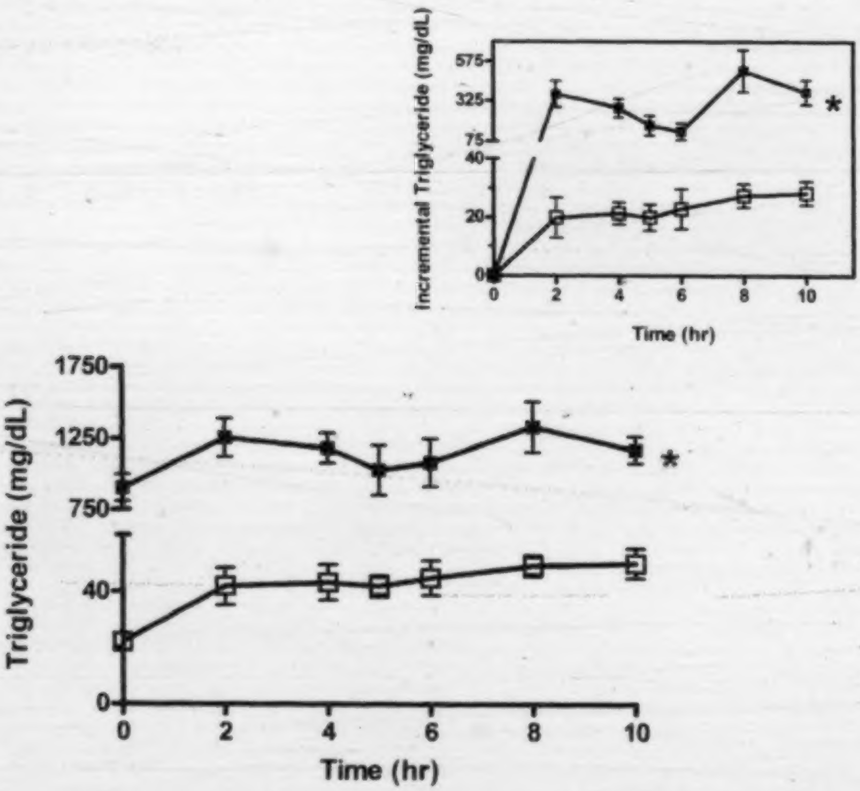

Figure 4-1. The postprandial response in plasma TG (AUC) following an oral fat challenge in the female JCR:LA-*cp* rats. Data are shown for PCOS *cp/cp* rats (filled squares) and control rats (open squares) as mean±S.E.M. The total AUC and the change in TG from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). The AUC for PCOS *cp/cp* animals is significantly greater than for control animals, (*) $p < 0.0001$. The iAUC for PCOS *cp/cp* animals is significantly greater than for control animals, (*) $p < 0.0001$.

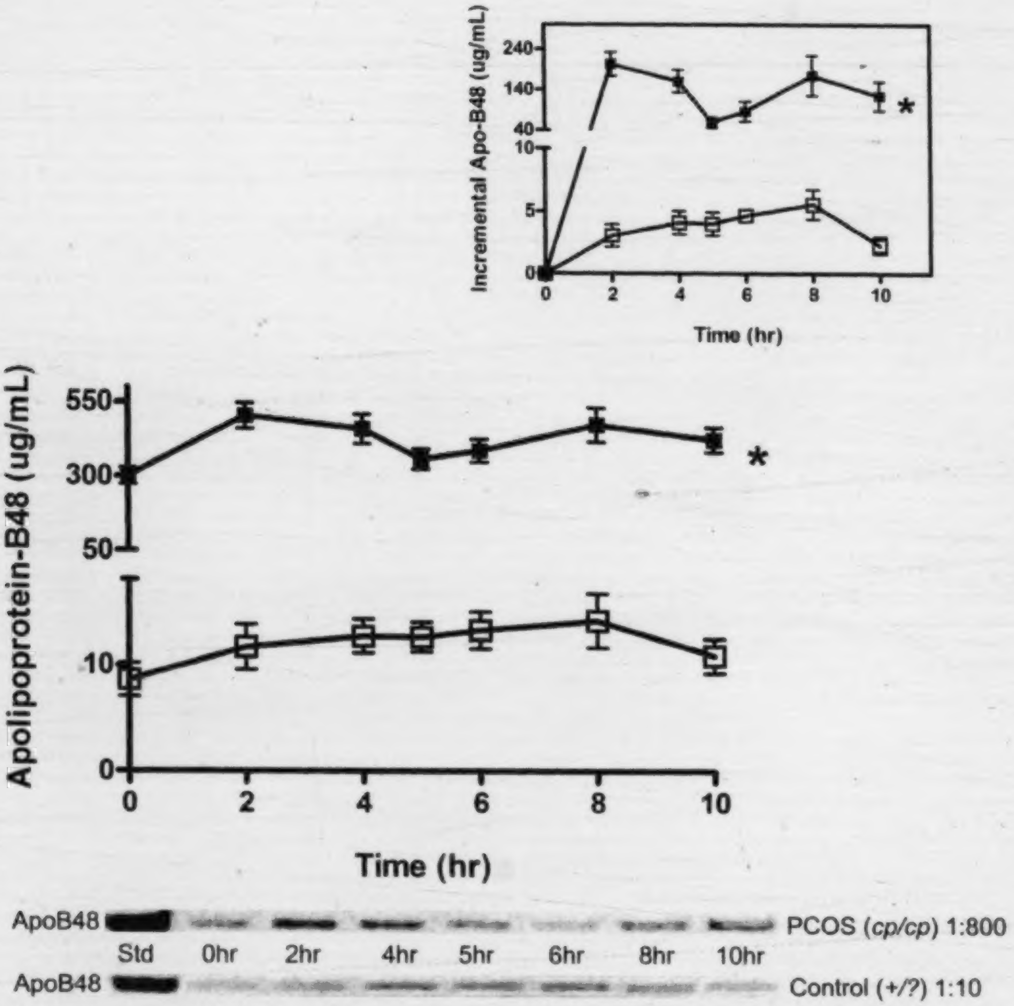

Figure 4-2. The postprandial plasma apoB48 response (AUC) following an oral fat challenge in JCR:LA-*cp* rats. Data are shown for PCOS *cp*/*cp* rats (filled squares) (*n* = 9) and control rats (open squares) (*n* = 9) as mean±S.E.M. The total AUC and the change in apoB48 from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). Both the postprandial apo-B48-AUC and the apoB48-iAUC was significantly greater in the PCOS *cp*/*cp* rats compared to control rats, (*) $p < 0.0001$. The lower panel represents a typical western blot detecting plasma apoB48 following an oral fat challenge (0–10 h) in both PCOS *cp*/*cp* (1:800) and control (1:10) JCR:LA-*cp* rats.

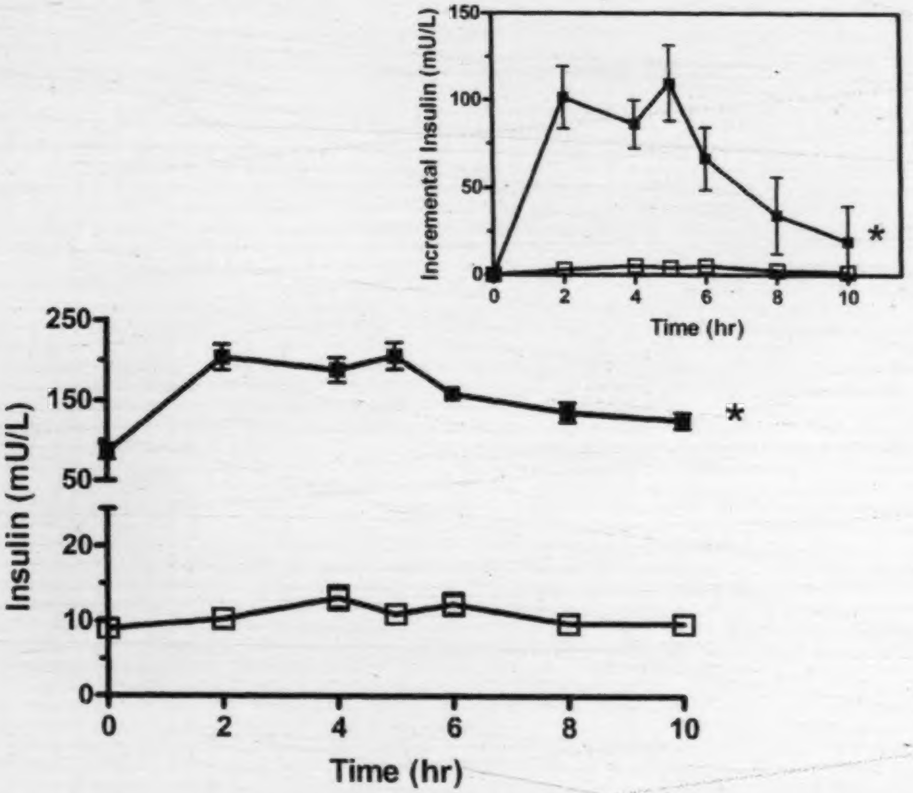

Figure 4-3. The postprandial response in plasma insulin following an oral fat challenge in JCR:LA-*cp* rats. Data are shown for PCOS *cp*/*cp* rats (filled squares) and +/? controls (open squares) as mean±S.E.M. The total AUC and the change (iAUC) in insulin from fasted concentrations is shown (inset). The PCOS *cp*/*cp* animals were shown to have a significantly greater total AUC and iAUC for plasma insulin following the oral fat challenge, (*) ($p < 0.0001$).

We explored that both fasting insulin and T concentrations were positively correlated with the postprandial response of both TG and apoB48. There was a further positive association between postprandial response of insulin (iAUC) and apoB48 (iAUC), consistent with previous findings (Vine *et al* 2007) (see Table 4-3).

Table 4-2. AUC and iAUC for triglyceride, apoB48 and insulin in the JCR:LA-*cp* rats.

	Control (+/?)	PCOS (*cp/cp*)
AUC_{TG}	429.8±39.3	10767.3±628.0^{a}
AUC_{apoB48}	122.9±17.9	4316.6±397.9^{a}
$AUC_{insulin}$	101.7±9.6	1627.4±49.2^{a}
$iAUC_{TG}$	209.0±35.4	2202.8±291.5^{a}
$iAUC_{apoB48}$	36.6±4.4	1327.1±194.9^{a}
$iAUC_{insulin}$	26.3±5.1	712.9±93.8^{a}

$^{a}p<0.0001$

Table 4-3. Pearson correlation coefficients for fasting and the change in the postprandial response (iAUC) for the JCR:LA-*cp* rats following an oral fat challenge.

	T	Insulin	Insulin^{iAUC}	TG	TG^{iAUC}	ApoB48	ApoB48^{iAUC}
T	-	.672^{b}	.793^{c}	.838^{c}	.676^{b}	.873^{c}	.860^{c}
Insulin	.672^{b}	-	.534^{a}	.723^{c}	.783^{c}	.822^{c}	.884^{c}
Insulin^{iAUC}	.793^{c}	.534^{a}	-	.880^{c}	.710^{c}	.837^{c}	.791^{c}

Values are in the fasted state (at baseline 0-h) unless otherwise stated; interpretation of indices in parentheses: T, testosterone; iAUC, incremental area under the curve between 0 and 10 h following oral fat challenge; TG, plasma triglyceride; apoB48, apolipoprotein B48; (–), not calculated.

$^{a}p < 0.05$.

$^{b}p < 0.01$.

$^{c}p < 0.001$.

Intestinal lymph CM Production and Lipid Content

The intestinal lymph CM production and content are shown in Table 4-4. Following administration of saline, defined here as the fasting state, the PCOS *cp/cp* animals secreted more than double the CM particles (measured by apoB48, one apoB48 is secreted/particle), compared to controls. The ratios of TC/apoB48 and TG/apoB48 reflect the proportion of TC and TG per CM particle, respectively. Interestingly, despite the increased particle number in the PCOS *cp/cp* rats, no difference was detected in the TC/apoB48 or TG/apoB48 ratio between the two groups.

As expected, following a lipid load, defined here as the postprandial state, both control and PCOS *cp/cp* animals had an increased secretion of CM particles (apoB48) compared to the fasting state. The PCOS *cp/cp* animals secreted more than double the CM particles (apoB48) compared to controls. The ratio of TC/apoB48 was twofold higher in PCOS *cp/cp* animals, however there was no difference in TG/apoB48 or particle size between two groups (Table 4-3).

ApoB48 secretion rate, both in fasting and postprandial states was positively correlated with fasting insulin concentrations (r=0.548, p=0.035 and r=0.565, p=0.044, respectively).

Table 4-4. The nascent intestinal lymph chylomicron content in the JCR:LA-*cp* rats.

	Saline		2% Intralipid	
	Control (+/?)	PCOS (*cp/cp*)	Control (+/?)	PCOS (*cp/cp*)
Triglyceride (mg/hr)	2.14±0.46	5.58±0.76[a]	13.78±3.58	29.21±2.40[b]
Total Cholesterol (mg/hr)	0.16±0.02	0.25±0.01[a]	0.16±0.05	1.03±0.12[b]
ApoB48 (ug/hr)	232.08±16.21	422.57±44.18[a]	408.74±40.69	808.40±89.37[b]
TC/apoB48	0.59±0.08	0.64±0.07	0.47±0.13	1.28±0.20[b]
TG/apoB48	8.47±1.53	11.04±1.57	32.72±5.44	36.58±4.40
Particle Size (nm)	130.00±5.47	131.91±4.89	183.50±8.37	197.13±6.84

Data shown are mean±S.E.M. for control and PCOS *cp/cp* animals
[a] $p<0.01$ vs. control rats, when given saline
[b] $p<0.01$ vs. control rats, when given 2% intralipid

4.4 Discussion

Dyslipidemia is a common clinical finding and postprandial dyslipidemia has also been reported in women with PCOS and the metabolic syndrome (Bahceci *et al* 2007). The aim of this study was to evaluate the postprandial lipid profile and the intestinal contribution to dyslipidemia in a rodent model of PCOS and the metabolic syndrome. Other androgen-induced rodent models of PCOS do not develop dyslipidemia and all features of the metabolic syndrome compared to the JCR:LA-*cp/cp* model of PCOS. The results of this study have shown for the first time that the JCR:LA-PCOS *cp/cp* rat has intestinal overproduction of CMs, and this contributes to the postprandial lipemia observed in these animals under the conditions of IR and hyperandrogenemia.

Results from both epidemiological (Boquist *et al* 1999, McNamara *et al* 2001, Karpe *et al* 2001) and experimental studies (Doi *et al* 2000, Yu *et al* 2001) have shown that postprandial lipemia and remnant lipoproteins, especially CMRs are risk factors for atherosclerosis and progression of coronary artery disease. Prolonged exposure to CMRs may increase the uptake of these particles by the arterial wall (Floren *et al* 1981, Proctor *et al* 2002), leading to atherosclerosis (Goldstein *et al* 1980, Nestel *et al* 1985). In disease states, such as hyperinsulinemia, the arterial wall appears to have exacerbated the uptake and retention of lipoprotein-derived cholesterol, particularly from CMRs (Zilversmit 1995, Huff 2003, Twickler *et al* 2005).

It is well established that women with PCOS often exhibit an atherogenic lipid and lipoprotein profile (Diamanti-Kandarakis 2007). In spite of numerous studies reporting dyslipidemia in PCOS, few studies have examined postprandial lipid metabolism. Velazquez *et al* (2000) observed that both lean and obese women with PCOS had an elevated postprandial TG response following a high fat meal (66% calories from fat), compared to healthy controls, and the postprandial hypertriglyceridemia was associated with IR (measured by oral glucose tolerance test) in these patients. Most recently, postprandial insulin/lipid response was evaluated in patients with PCOS (Bahceci *et al* 2007) and showed that PCOS patients with IR had an increased postprandial response of TG, TC, and total apoB, following a high fat diet (65% calories from fat) compared to age-and BMI-matched controls. Collectively, the high prevalence of both IR and postprandial dyslipidemia may add further risk of CVD development in PCOS. In this study, we used a novel OFC test analogous to the existing approach used in human studies (Tagart *et al* 1997, Vine *et al* 2007, Bahceci *et al* 2007). We demonstrated that PCOS *cp/cp* rats developed postprandial dyslipidemia, with an increased response of TG and apoB48, as observed in human studies (Bahçeci *et al* 2007, Velazquez *et al* 2000). These results suggest that the postprandial lipemia and elevated apoB48 concentrations may contribute to early atherogenesis and cardiovascular dysfunction in this rodent model (Russell *et al* 1998, O'Brien 2000).

The underlying pathphysiology of dyslipidemia in PCOS is not clear. IR has been recognized as a common component of PCOS and the metabolic syndrome, and the association between IR and dyslipidemia is well established (Legro *et al* 2001, Velazquez *et al* 2000). In this study, we have shown that the postprandial response of plasma TG ($iAUC_{TG}$) was positively associated with fasting plasma insulin levels, as observed in human studies (Legro *et al* 2001, Velazquez *et al* 2000, Lewis *et a*l 1991, Tobey *et al* 1981, Chen *et al* 1993, Katzel *et al* 1994). The postprandial response of plasma CM ($iAUC_{apoB48}$) was found to be positively associated with fasting plasma insulin levels in the JCR:LA-*cp* rats, which has not been widely investigated in human studies. We have also shown that the postprandial insulin response ($iAUC_{insulin}$) is associated with postprandial TG and apoB48 responses, which is consistent with a modulatory role of insulin on postprandial dyslipidemia in PCOS. However, whether insulin directly modulates the synthesis and secretion of intestinal lipoproteins or regulates the clearance of TG-rich lipoproteins in this model of PCOS is not fully understood. Previous evidence in insulin resistant hamsters has demonstrated that intestinal lipoprotein concentrations are increased (Federico *et al* 2006, Haidari *et al* 2002), and lipoprotein lipase activity is decreased (Potts *et al* 1995), which may be analogous to the human condition of PCOS and the metabolic syndrome (Lithell *et al* 1987).

Our study has shown that the postprandial response of apoB48 and TG (both AUC and iAUC) are increased in PCOS *cp/cp* animals, compared to control animals. The accumulation of postprandial TG and CMs can be attributed to an increased intestinal production as evidenced by the intestinal lymph data in PCOS *cp/cp* phenotype. However, a decreased clearance of CMs may exacerbate postprandial lipemia and this is under further investigation. Increasing evidence has demonstrated that intestinal lipoprotein secretion may be a prominent contributor to the fasting and postprandial dyslipidemia in the insulin resistant state (Phillips *et al* 2002a, b, Vine *et al* 2007, Haidari *et al* 2002, Duez *et al* 2006). Recently, Duez *et al* (2006) showed that hyperinsulinemia was associated with increased production of intestinal apoB48-containing lipoproteins in humans (using a primed constant (12 h) infusion of deuterium-labeled leucine) which is consistent with our findings in the PCOS *cp/cp* model. We found that the apoB48 secretion rate into the lymph, both in the fasting and postprandial state was positively correlated with fasting insulin concentrations. Presently, the mechanisms of intestinal CM, especially apoB48 assembly and secretion in the enterocytes, and their association with IR are not fully understood (Hussain 2000, Federico *et al* 2006). Studies on both human and chow-fed animals suggested that insulin has an acute inhibitory effect on apoB48 secretion, when the enterocytes are insulin sensitive (Haidari *et al* 2002, Federico *et al* 2006, Allister *et al* 2006). However, in conditions of IR, the intestine is no longer sensitive to the down-regulatory effects of insulin on apoB48 (Federico *et al* 2006, Haidari *et al* 2002), and the secretion of apoB48 is increased, as observed in this study . Interestingly, we have also shown that in the postprandial state, the CMs of the PCOS *cp/cp* rats contained two-fold higher TC, compared to control animals. This finding may suggest that the postprandial CMs from the PCOS *cp/cp* animals are more atherogenic, due to an increased capacity to deliver cholesterol to the arterial wall (Proctor *et al* 2002).

Testosterone may play a role in regulating lipid metabolism. In the present study, we have shown that T concentration is positively correlated with fasting insulin and the postprandial response of TG and apoB48 (iAUC). However, it is not clear if T has direct effects on lipid metabolism or if the dyslipidemia observed is a result of altered insulin metabolism in this model. Lithell *et al* (1987) demonstrated decreased postprandial LPL activity in the adipose tissue of PCOS women, which was inversely correlated with T concentration. It has also been reported that hyperandrogenemia is associated with increased catecholamine-induced lipolysis in adipocytes and release of free fatty acids (FFAs) from adipose tissue into circulation (Diamanti-Kandarakis 2008). A recent human study on non-PCOS subjects by Duez *et al* (2008) has shown that acute elevation of free fatty acids (FFAs) can up-regulate intestinal CM production. In addition, T may act through the androgen receptor to down-regulate insulin signalling in peripheral tissues (Corbould *et al* 2007, Allemand *et al* 2005), and thus has been proposed to induce androgen receptor-mediated IR in PCOS. Therefore, increased T may lead to or exacerbate IR and indirectly effect lipid metabolism (Diamanti-Kandarakis 2008).

4.5 Conclusions

The PCOS *cp/cp* rats demonstrated postprandial dyslipidemia, which was positively correlated with T and fasting insulin concentrations. In this rodent model of PCOS and the metabolic syndrome, an increase in intestinal CM production contributes to hypertriglyceridemia and postprandial dyslipidemia. The JCR:LA-*cp/cp* rat represents a potentially useful model to study the early development of dyslipidemia and CVD risk in the context of hyperandrogenimia and IR, in particular postprandial lipid metabolism and CVD risk in PCOS.

4.6 Literature Cited

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5. General Summary and Discussion

5.1 Summary of Specific Objectives and Results

1. The female JCR:LA-*cp/cp* rats have hyperandrogenemia, a key clinical feature of PCOS.

This hypothesis is supported by the results shown in chapter 3. The PCOS *cp/cp* animals tended to have increased T at the age of 6wks. At 12wks of age, the PCOS *cp/cp* animals had a 70% increase in T concentration, compared to control animals. The concentration of T was positively correlated with fasting plasma insulin concentration.

2. The female JCR:LA-*cp/cp* rats demonstrate ovulatory dysfunction and abnormal ovarian follicular development.

The results in chapter 3 support this hypothesis. The PCOS *cp/cp* animals developed oligo-ovulation and irregular estrous cycle monitored by daily vaginal smear. The follicular development of the PCOS *cp/cp* animals was abnormal, characterized by an increased number of total and atretic/cystic follicles, decreased proportion of secondary follicles and an increased proportion of tertiary follicles. The proportion of late tertiary follicles also tended to decrease in the PCOS *cp/cp* animals. Collectively, these results suggest that early follicular growth was increased, and the follicular development arrested at the early tertiary stage in the PCOS *cp/cp* animals.

3. The female JCR:LA-*cp/cp* rats develop obesity, IR and dyslipidemia, as observed in human PCOS.

This hypothesis is supported by the results reported in chapter 3. The PCOS *cp/cp* animals demonstrated obesity, IR and fasting dyslipidemia (increased plasma TG, TC and apoB48 concentrations) at 6wks of age, and these metabolic abnormalities were exacerbated at the age of 12wks. Fasting TG and apoB48 concentrations were positively correlated with insulin and T concentrations, suggesting an associated and possible interactive role of insulin and T in the development of dyslipidemia in the JCR:LA-*cp/cp* rodent model of PCOS.

4. The JCR:LA-*cp/cp* rats present with postprandial dyslipidemia, and the intestinal overproduction of lipoproteins contributes significantly to this dyslipidemia.

The results in chapter 4 support this hypothesis. We observed that the PCOS *cp/cp* genotype demonstrated postprandial dyslipidemia with an increased response of TG and apoB48 following an OFC, and this was positively correlated with fasting insulin concentrations. The results from the lymph cannulation studies showed that the PCOS *cp/cp* animals secreted more CM particles (apoB48), TG and TC in both the fasting and postprandial states. The CMs secreted in the PCOS *cp/cp* genotype in the postprandial state contained a twofold higher TC compared to control animals, which suggests that postprandial CMs in the PCOS *cp/cp* animals may be more atherogenic.

5.2 General Discussion

The overall aim of this study was to characterize and establish the female JCR:LA-*cp/cp* rat as a model of PCOS in the context of the metabolic syndrome, and to evaluate CVD risk factors, including IR and dyslipidemia in this model. Although PCOS has been studied for more than 70 years, the etiology of PCOS and the pathophysiological mechanisms associated with the reproductive and metabolic disturbances in PCOS remain unclear. To date, several rodent models have been established to explore the pathogenesis and to determine the early etiology of PCOS, however no model presents with all the symptoms of PCOS, in particular the metabolic abnormalities (Table 1-6).

The results of this thesis have shown that there are several advantages of the JCR:LA-*cp/cp* rats compared to other rodent models of PCOS. Firstly, the JCR:LA-*cp/cp* rat is a spontaneous model, in that it develops features of PCOS without androgen or other induction treatments. Presently, most rodent models of PCOS require invasive treatments to induce the PCOS condition and interfere with the hypothalamic-pituitary-ovarian axis to cause an acute state of PCOS. Secondly, the JCR:LA-*cp/cp* rats develop the common metabolic disorders observed in PCOS women, including obesity, IR and dyslipidemia, particularly postprandial dyslipidemia (Norman *et al* 2007, Diamanti-Kandarakis *et al* 2008, Bahceci *et al* 2007). Although the metabolic syndrome is very common in PCOS, and about 90% of women with PCOS have at least one metabolic disorder (Essah *et al* 2007), few studies have focused on the metabolic abnormalities in rodent models of PCOS. The first rodent model reported to present with both PCOS and metabolic changes (increased body weight, increased body fat, and enlarged mesenteric adipocytes, as well as elevated leptin levels and IR) was DHT-induced (Manneras *et al* 2007). However, the DHT-induced PCOS model had a normal lipid profile, which may not represent up to 70% of PCOS women with dyslipidemia. In addition, postprandial dyslipidemia is related to the premature development of atherosclerosis (Twickler *et al* 2005), and has been reported in women with PCOS (Bahceci *et al* 2007). Thirdly, the JCR:LA-*cp/cp* rats develop PCOS and the metabolic syndrome gradually, similar to the chronic course of PCOS from adolescence to adulthood in women. The PCOS *cp/cp* animals demonstrated hyperandrogenemia and metabolic disorders as early as 6wks of age and progressively these were exacerbated at the age of 12wks. Therefore, it is a useful model to study the early development of CVD risk factors and the effects of early nutritional and pharmaceutical intervention in PCOS. Finally, the JCR:LA-*cp/cp* rat is the only animal model of PCOS that can be used to test the hypothesis that hyperinsulinemia leads to hyperandrogenemia. Indeed, in this model hyperinsulinemia precedes hyperandrogenemia. The JCR:LA-*cp/cp* rats have dysfunction of the leptin receptor, which is similar to leptin resistance and hyperleptinemia observed in obese PCOS women. It can be hypothesized that in this model, leptin resistance leads to obesity, IR and compensatory hyperinsulinemia (Kieffer *et al* 1996, Goumenou *et al* 2003), resulting in hyperandrogenemia and ovulatory dysfunction. In summary, the JCR:LA-*cp/cp* rat is a novel and useful model to study the pathogenesis of PCOS, particularly the relationship between reproductive and metabolic disorders, and the development of CVD. A summary of the reproductive and metabolic abnormalities in PCOS women, the JCR:LA-*cp/cp* rats and DHT-induced rats is shown in Table 5-1.

The JCR:LA-*cp/cp* rodent model of PCOS does have some shortcomings which need to be discussed. PCOS is a heterogeneous disorder reflecting multiple etiologies involving both environmental and genetic factors (Franks *et al* 2006). Although obesity and IR are very common in PCOS, there are still PCOS women without obesity or IR. The etiology of PCOS in these women may be and is likely to be different from that of the obese and insulin resistant women. Therefore, the JCR:LA-*cp/cp* rats may only represent one subtype of PCOS. In this study, the JCR:LA-*cp/cp* rat develop severe hypertriglyceridemia, with concentrations more than 40 times greater than those of control animals. In fact, this severe hypertriglyceridemia is rarely seen in human PCOS. It is possible that such high concentrations of TG may exacerbate the metabolic and reproductive symptoms and increase the risk of CVD. Furthermore, we have shown that the ovaries from PCOS *cp/cp* rats demonstrate clusters of lipid droplets, which have not been observed in human PCOS. The pathophysiological mechansims of how these lipid droplets are formed and how they may affect ovarian function (endocrine hormone synthesis and folliculargenesis) remains unknown, but they may be associated with the severe hypertriglyceridemia observed in the PCOS *cp/cp* rats.

Table 5-1. Summary of reproductive and metabolic abnormalities in PCOS women, the JCR:LA-*cp/cp* rats and DHT-induced rats.

	PCOS women	the JCR:LA-*cp/cp* rats	DHT-induced rats
Reproductive			
Testosterone	↑	↑	-
Estradiol	↑/-	↑/-	-
Ovulation disturbances	Yes	Yes	Yes
Ovarian weight	↑	↓	↓
No. of follicles	↑	↑	↑
Follicular cysts	Yes	Yes	Yes
Follicular atresia	Yes	Yes	Yes
Granulosa layers	↓	↓	↓
Metabolic			
Obesity	Yes	Yes	Yes
Insulin sensitivity	↓	↓	↓
Leptin	↑	↑	↑
Dyslipidemia			
Fasting dyslipidemia	Yes	Yes	No
Postprandial dyslipidemia	Yes	Yes	Unknown
Early cardiovascular impairment	Yes	Yes	Unknown

↓, Decrease; ↑, increase; —, no change

Additionally, we also found that the ovaries from the PCOS *cp/cp* rats demonstrated mild to moderate morphological change compared to other animal models of PCOS (Manneras *et al* 2007, McCarthy *et al* 1990). The ovarian weight of the PCOS *cp/cp* animals was decreased and the cystic follicles were not as frequent or as large as those of other induced rodent models of PCOS. In the JCR:LA-*cp/cp* rodent model of PCOS, hyperinsulinemia appears to be the primary factor leading to hyperandrogenemia and ovarian morphological change, which takes a chronic and progressive course. It is hypothesized that older animals may present with more severe ovarian pathophysiology.

A number of unanswered questions have been generated from this study and provide interesting directions for future research on the mechanisms associated with early and progressive CVD development in this spontaneous model of PCOS and the metabolic syndrome.

PCOS is an androgen excess disorder, and androgens play a significant role in follicular development and ovarian dysfunction in PCOS. Hyperandrogenemia is also believed to contribute to the metabolic abnormalities in PCOS. However, the secretion and metabolism of androgens are directly regulated by the hypothalamic-pituitary-ovarian axis. The pituitary gonadotroph plays a central role in reproductive function by secreting LH and FSH (Balen 2004). LH has a vital role in the follicular phase, inducing thecal androgen production and initiating oocyte maturation at midcycle. FSH is the initial stimulus for follicular development and also conversion of androgens to estrogens by stimulating the aromatase enzymes in granulosa cells. The production and secretion of FSH and LH is directly under the influence of hypothalamic GnRH. When the GnRH pulsatility is rapid, LH secretion predominates and the ratio of LH/FSH is increased, which favors the overproduction of androgens in the ovarian theca cells (Haisenleder *et al* 1991). We have shown that the JCR:LA-*cp/cp* rats develop hyperandrogenemia with normal E_2 levels, consistent with human PCOS, however the function of the hypothalamic-pituitary-ovarian axis has not been evaluated in this rodent model of PCOS. In addition to the hypothalamic-pituitary-ovarian axis, several hormones are also involved in regulating androgen secretion. Inhibin B and antiMüllerian hormone (AMH) have negative effects on FSH production/bioactivity, and indirectly promotes androgen synthesis (Tsilchorozidou *et al* 2004, Pellatt *et al* 2007). Activin opposes the effects of inhibin B (Norman *et al* 2001). Therefore, future studies may focus on the function of hypothalamic-pituitary-ovarian axis and hormone profile to explore the pathophysiology of PCOS in the JCR:LA-*cp/cp* rats.

Future studies could explore the role of hyperandrogenemia in the development of dyslipidemia and CVD risk factors in PCOS. PCOS has been identified as an androgen excess disorder (Azziz *et al* 2006), associated with features of the metabolic syndrome, including IR, obesity and dyslipidemia, which are risk factors for CVD. However, the exact role of androgens in the development of these CVD risk factors, particularly the onset of CVD in PCOS is not clear (Diamanti-Kandarakis *et al* 2008). In this study, we have shown that T concentration is positively correlated with fasting insulin, TG and apoB48 concentrations in the JCR:LA-*cp* rats. In fact, hyperandrogenism, hyperinsulinemia, obesity and dyslipidemia are intimately related, and hyperandrogenemia has been implicated as a negative mediator and/or exacerbator of the metabolic disorders in PCOS (Essah *et al* 2007). T has been shown to induce androgen receptor-mediated IR in peripheral tissues, which was selective for metabolic signaling pathways (Cordbould 2007). In adipose tissue, T acts via decreased protein kinase Cξ (PKCξ) (Cordbould 2007), and in skeletal muscle, it acts via increased mammalian target of rapamycin (mTOR) and ribosomal S6-kinase (S6K) (Allemand *et al* 2005). The androgen receptor-mediated IR may be the key factor linking hyperandrogemism and the metabolic disorders together (Diamanti-Kandarakis *et al* 2008). Additionally, hyperandrogenism in PCOS may favor visceral obesity, which is linked to IR and dyslipidemia. Androgens are also shown to be involved in the regulation of human LPL activity (Lithell *et al* 1987). In adipose tissue of women with PCOS, an inverse relation between T concentration and LPL activity was demonstrated. However, it is still debated whether PCOS, an androgen excess disorder is a risk factor of CVD, independent of the metabolic abnormalities. Over recent decades, the gender disparity in susceptibility to CVD has been attributed to the difference in sex steroids with estrogens as a cardioprotective factor and androgens as a possible exacerbator of CVD (Liu *et al* 2003). However, no study in men or women without PCOS has shown that androgen status plays a significant role in the development of CVD (Wu *et al* 2003, Barrett-Connor *et al* 1995). Moreover, in pre- and post menopausal women carotid intima-medial thickness (CIMT) has been shown to be inversely correlated with endogenous DHEAS and T (Bernini *et al* 1999), suggesting that androgens may have some protective effects against CVD. However, an animal experiment showed that administration of T to female primates was associated with increased atherogenesis, independent of lipid profile (Adams *et al* 1995). Unfortunately, similar experiments in animal models of PCOS have not been reported to date. It is proposed that administration of androgens to control animals (+/?) would help to explore the role of androgens in the development of PCOS, CVD risk factors and the onset of CVD.

Future studies may also focus on age-tracking animals to monitor the progression of the metabolic and reproductive disorders, the development of early cardiovascular impairment and the onset of CVD in the JCR:LA-*cp/cp* rodent model of PCOS. It is now recognized that PCOS has a peri-menarcheal onset with the maturation of hypothalamic-pituitary-ovarian axis (Franks 2008). Similar to the pattern seen in young adult women, PCOS is a common cause of menstrual irregularities in adolescent girls (Buggs *et al* 2005, Warren-Ulanch *et al* 2006), associated with features of the metabolic syndrome, including obesity, IR and dyslipidemia (Essan *et al* 2007). It has been estimated that the prevalence of metabolic syndrome among adolescent girls with PCOS is 19.4%, a rate 3-fold higher than BMI and ethnicity-adjusted controls using the Third National Health and

Human Examination Survey III (NHANES III) data (Leibel *et al* 2006). Therefore, adolescent girls with PCOS, similar to adult women with the condition, are at increased risk for the development of type 2 diabetes mellitus and CVD, compared to the general adolescent population (Essan *et al* 2007). It is important to recognize girls and young women at risk for PCOS, as early intervention may prevent long term health outcomes and improve quality of life (Trent *et al* 2002). We have shown that the 6-week-old JCR:LA-*cp/cp* rats (adolescent) develop mild hyperandrogenemia, with obesity, IR and dyslipidemia, as observed in adolescent girls with PCOS. Indeed, our preliminary studies have shown that the adult female JCR:LA-*cp/cp* rats have increased LV mass, reduced % ejection volume and % fractional shortening, which are associated with LV dysfunction, compared to control animals (unpublished data). Our initial vascular reactivity studies using aortic rings have also shown that the female JCR:LA-*cp/cp* rats have impaired endothelial function, characterized by impaired vasodilation in response to acetylcholine (Ach), and an exacerabated relaxation response in the presence of the NO donor sodium nitroprusside (SNP) (unpublished data). Therefore, the JCR:LA-*cp/cp* rat is a potential model to study early development and the long term CVD outcomes in PCOS, and to explore effective treatments in early life stages to prevent the progressive development of CVD in this syndrome.

Previous studies have focused on the role of liver and adipose tissue in dyslipidemia in PCOS. In this study, we have demonstrated that intestinal overproduction of apoB48-containing lipoproteins contributes significantly to dyslipidemia, particularly postprandial dyslipidemia in the JCR:LA-*cp/cp* rats, which may be another possible mechanism of dyslipidemia in PCOS (Fig 5-1). We have also shown that intestinal apoB48 secretion rate, both in the fasting and postprandial state was positively correlated with fasting insulin concentrations in the JCR:LA-*cp* rats, which suggests unresponsiveness of intestinal lipoprotein production to the inhibitory effects of insulin and the development

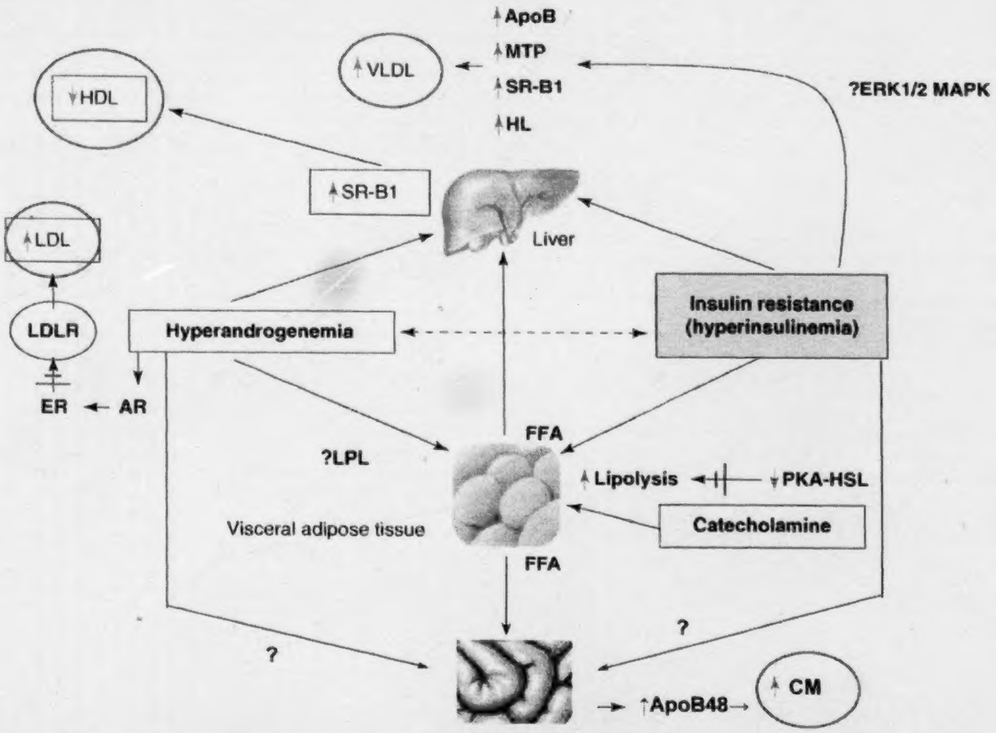

Figure 5-1. Possible mechanisms of dyslipidemia in PCOS. Within adipocytes, IR and hyperandrogenemia result in increased catecholamine-induced lipolysis and release of fatty acids into circulation. Increased free fatty acid flux to the liver stimulates the assembly and secretion of VLDL resulting in hypertriglyceridemia. In an insulin resistant state, intestinal lipoprotein secretion may be increased. Increased FFA in circulation may also contribute to intestinal overproduction of apoB48-containing CMs. The main serum lipid abnormalities in PCOS are indicated in circles. Broken arrow represents potential interaction, ↑activation; ↓deactivation; ≠, inhibition. Abbreviations: ApoB, apolipoprotein b; AR, androgen receptor; ER, estrogen receptor; ERK1/2 MAPK, extracellular signal-regulated kinase 1/2 mitogenactivated protein kinase; FFA, free fatty acids; HL, hepatic lipase; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; MTP, microsomal triglyceride protein; PKA-HSL, protein kinase a-hormone sensitive lipase complex; SR-B1, scavenger receptor-b1; VLDL, very low density lipoprotein; CM, chylomicron; ApoB48, apolipoprotein B48. Adapted from Diamanti-Kandarakis *et al* 2007.

of IR at the level of the intestine. In addition, IR and hyperandrogenemia may result in increased catecholamine-induced lipolysis and release of fatty acids from adipose tissue into circulation. Interestingly, a recent human study on non-PCOS subjects by Duez *et al* (2008b) has shown that acute elevation of free fatty acids (FFAs) can stimulate intestinal apoB48-containing lipoprotein production. Therefore, it is hypothesized that when exposed to hyperinsulinemia, insulin resistant enterocytes have increased rates of *de novo* lipogenesis and lipoprotein assembly and secretion, which is exacerbated in the presence of increased FFA (Duez *et al* 2008a). However the molecular basis linking IR and intestinal overproduction of lipoproteins is unknown and the direct effect of hyperandrogenemia on intestinal overproduction of lipoproteins has not been investigated in this model, which will be explored in future studies.

Other studies could evaluate the atherogenicity of CMs isolated from the PCOS genotype, and the capability of the arterial wall to uptake the CMRs in the PCOS condition. In this study, we have shown that the PCOS *cp/cp* genotype develops dyslipidemia, including postprandial dyslipidemia, which is in part due to the intestinal overproduction of lipoproteins. It is well known that both the content of the CMs and metabolic condition, such as IR contribute to the development of atherosclerosis (Proctor *et a*l 2002). The combination of dyslipidaemia and IR would suggest that women with PCOS might have accelerated vascular disease. Indeed, vascular morphological studies have shown that CIMT and coronary artery calcification are increased in PCOS women, which are indications of subclinical atherosclerosis, and reflect the underlying degree of atherosclerosis (Guzick *et al* 1996, Talbott *et al* 1998, Christian *et al* 2000). Eighty percent of obese PCOS women have IR (Dunaif 1997), and studies have indicated that the arterial wall may exacerbate the uptake of the cholesterol-rich lipoproteins, including CMRs in insulin-resistent state, contributing to atherosclerosis (Camejo *et al* 2002, Raines *et al* 2005). However, we do not know whether this is the case in PCOS. The postprandial intestinal CMs produced by the PCOS *cp/cp* animals contained twofold more cholesterol compared to controls. Therefore, it may be hypothesized that the CMRs derived from the CMs of the PCOS *cp/cp* animals are more atherogenic. Future studies in the PCOS *cp/cp* animals could examine the plasma clearance and arterial uptake of

CMRs isolated from these animals to examine the atherogenicity of these particles.

Lastly, our future studies may assess the effect of lifestyle modification (nutrition, weight loss) and pharmaceutical impact of insulin-sensitizer (metformin and thiazolidinediones) and anti-androgen medications (spironolactone and flutamide) on the progression of cardiovascular risk factors and the development of hyperadrogenemia and CVD in the JCR:LA-*cp/cp* rodent model of PCOS. The association between obesity, hyperandrogenemia, IR, menstrual abnormalities, and infertility has emphasized the need to address lifestyle modification and insulin sensitizing treatment in women with PCOS. Studies have shown that the loss of just 5% or more of body weight by lifestyle modification (exercise and dietary intervention) can ameliorate the symptoms of PCOS, including reduced severity of hirsutism and acne, and restoration of menstrual regularity and ovulation (Kiddy *et al* 1992, Pasquali *et al* 1989). Dietary recommendation for PCOS women includes high-fiber complex carbohydrates, moderate levels of protein, and adequate fat to meet essential fatty acid needs (Moran *et al* 2008). Diet containing ω-3 fatty acids and monounsaturated fatty acids (MUFA) with limited amounts of trans and saturated fatty acids has also been recommended (Moran *et al* 2008). However, few studies have examined the effects of dietary intervention on long term health problems, in particular CVD in PCOS.

Although it is not currently licensed, insulin sensitizers have been widely used to reduce insulin and androgen concentrations and to treat anovulation associated with PCOS (Homburg 2008). Metformin acts by decreasing hepatic glucose production, improving glucose utilization in the periphery, reducing intestinal glucose uptake, and decreasing lipolysis (Morin-Papunen *et al* 1998). Thiazolidinediones including pioglitazone and rosiglitazone improve the peripheral action and utilization of insulin (Romualdi *et al* 2003). Additionally, some evidence has suggested that metformin and thiazolidinediones may act directly to attenuate ovarian steroidogenesis (Cibula *et al* 2005, Seto-Young *et al* 2005). Indeed, studies have demonstrated that treatments with insulin sensitizers improve both the metabolic and reproductive symptoms of PCOS (Baillargeon *et al* 2004, 2006). In addition, metformin has been shown to be beneficial in improving forearm vascular reactivity response and endothelial function in PCOS (Diamanti-Kandarakis *et al* 2005, Orio *et al* 2005), which may be due to its effects on suppression of circulating androgens, and modulating inflammation of cytokines (McCarthy *et al* 2004). However the long term beneficial effects of insulin sensitizing treatment on sustaining improvements in endothelial function, inflammatory status, or modulating left LV function and its impact on CVD events remain unknown.

Anti-androgen drugs including flutamide and spironolactone have been mainly administered to women with hirsutism and acne (Homburg 2008). Flutamide is a non-steroidal anti-androgen that inhibits DHT binding to the androgen receptors. Spironolactone is an aldosterone antagonist competitively inhibiting the binding of T and DHT to the androgen receptor. However the effects of these anti-androgen treatments on metabolic disorders in PCOS, particularly IR are contradictory, with partially reverse of peripheral IR associated with hyperandrogenism (Moghetti *et al* 1996) versus no effect on IR in women with PCOS (Diamanti-Kandarakis *et al* 1995, Dunaif *et al* 1990). The JCR:LA-*cp/cp* model could be used to examine the potential effects of these agents in ameliorating hyperandorgenemia, reproductive function and cardiometabolic indices. They could also be used to examine the mechanisms of insulin/hypernadrogenemia in the development of PCOS.

In summary, the findings in this thesis supports the JCR:LA-*cp/cp* rat as a model of PCOS and the metabolic syndrome. Further studies could be directed to focus on the mechanisms involved in the development of PCOS, the relationship between CVD risk factors and hyperandrogenemia, and the effects of early interventions on the progression of PCOS and CVD risk using this unique rodent model of spontaneous PCOS.

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